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14. ABSTRACT In this funding period, initiating PI and partnering PI collaborated to test the hypothesis that silencing of key tumor suppressive genes by enhanced crosstalk between LSD1 and HDAC is a unique epigenetic mechanism promoting TNBC growth, and blockade of the HDAC5-LSD1 axis results in profound inhibition of TNBC growth and metastasis. In this funding cycle, we found that the expression levels of LSD1 and HDAC5 are negatively associated with T-cell attracting chemokines and PD-L1 in clinical TNBC specimens. Further study found that Inhibition of LSD1 significantly increased expression of T-cell chemokines and PD-L1 in TNBC cell lines. Combination of LSD1 inhibitor and PD-1/PD-L1 blockade exhibited significant synergy in hindering breast cancer progression. We also explore the roles of LSD1 homolog, LSD2 (KDM1B), in breast oncogenesis. We found that overexpression of LSD2 protein significantly altered expression of key important epigenetic modifiers such as LSD1, HDAC5, etc., promoted cellular proliferation and augmented formation of larger colonies in soft agar. We also demonstrated that LSD2 overexpression in MDA-MB-231 cells facilitated mammosphere formation, enriched CD49f+/EpCAM- subpopulation and induced expression of pluripotent stem cell markers Nanog and Sox2. Stable overexpression of LSD2 resulted in accelerated growth of MDA-MB-231 xenograft tumors in nude mice. The interactive studies between two PIs have resulted in one conference abstract presented at AACR annual meeting and a research article published in *Oncotarget*. Several manuscripts are currently being prepared for publication.

15. SUBJECT TERMS Nothing listed

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Table of Contents

1. INTRODUCTION

Triple negative breast cancer (TNBC) is clinically classified based on the absence of the estrogen receptor, the progesterone receptor, or HER2 receptor overexpression. TNBC is more aggressive and distant metastases to lung, liver, brain and bone are more common in TNBC patients than their hormone receptor-positive counterparts. Therefore, new targeted approaches are urgently needed to improve TNBC treatment and prevention. This funded Breast Cancer Breakthrough Award is initially a partnership between Dr. Yi Huang (initiating PI) and Dr. Nancy E. Davidson (partner PI). During the current funding period, Dr. Nancy E. Davidson moved to University of Washington at Seattle and transferred the Partnering PI to Dr. Steffi Oesterreich at University of Pittsburgh. Drs. Huang and Oesterreich continued to test the hypothesis that crosstalk between HDAC and LSD1 leads to the initiation and progression of TNBC, and addressed how HDAC-LSD1 interaction suppresses expression of tumor suppressor genes (TSGs) to facilitate TNBC development. Our research papers reporting these novel findings were recently published in *Oncogene* (2017 Jan 5;36(1):133-145, PMID: 27212032, final version) and *Oncotaget* (2017, July 19, DOI: 10.18632/oncotarget.19387).

2. KEYWORDS

TNBC, HDAC5, LSD1, LSD2, immunotherapy, HCI-2509, combination therapy

3. ACCOMPLISHMENTS

a. What were the major goals of the project?

The main aims of this proposal are to: (1) elucidate how changes in interaction of HDAC5 and LSD1 contribute to TNBC initiation and progression; and (2) evaluate the *in vivo* roles of HDAC5-LSD1 axis in TNBC development and test the combinatorial effect of novel inhibitors of HDAC5 and LSD1 in TNBC therapy.

b. What was accomplished under these goals?

Proposed Aims (3rd year)	Accomplishment
Specific Aim 3: Evaluate therapeutic effects of combination strategies in patient-derived xenografts.	The Huang lab has assisted Dr. Oesterreich lab to evaluate the therapeutic effect of combination strategy targeting LSD1/HDACs axis using the PDX model to aid in establishing clinical relevance in preparation for design of clinical trials and assess the effects of combination treatment on proliferation markers, histone modifications and expression of aberrantly silenced genes in PDXs (**Fig. 1**). Please review Dr. Oesterreichôs separate report for detailed results and accomplishment.
Other reportable results	In this funding cycle, we found that inhibition of LSD1 induces T-cell chemokine and PD-L1 expression in TNBC cells. We assessed the effect of LSD1 on expression of key T-

	cell attracting chemokine genes, and showed that treatment with LSD1 inhibitor HCI-2509 significantly induced expression of chemokines CCL5, CXCL9, and CXCL10 in breast cancer MDA-MB-231 cells (**Fig. 2A**). Similar to pharmacological effect of LSD1 inhibition, siRNA mediated LSD1 depletion also boosted the expression of these chemokines (**Fig. 2B**). Next, the effect of HCI-2509 on the CD8+ T cell infiltration in tumor microenvironment was examined through ex vivo chemotaxis assay. CD8+ T cells were purified from splenocytes of BALB/c mice, and expanded in low dose IL-2 with anti-CD3/anti-CD28 activation. The activated CD8+ T cells were placed in upper chamber in 24-well plates containing mouse breast cancer cell 4T1 at bottom. Cells were treated with vehicle or HCI-2509. After incubation, cells in the bottom well were collected and counted by flow cytometry for CD8+ population (**Fig. 2C**). The result showed that HCI-2509 significantly augmented the presence of CD+8 T cells (**Fig. 2D**). We further studied the combinatorial effect of LSD1 inhibitor, HCI-2509, and small molecule of PD-1/PD-L1 inhibitor, I2, on proliferation of normal breast epithelial MCF-10A cells versus MCF-10A-CA1a cells, transformed malignant line from parental cells. The combination indexes (CI) were calculated using the Chou-Talalay model (13). Co-treatment with HCI-2509 and I2 led to a strong synergistic effect (CI < 1) in hindering MCF-10A-CA1a growth, however additive or antagonistic effect (CI Ó 1) was found in MCF-10A cells (**Fig. 3A**). Further study showed that stable knockdown of LSD1 by shRNA sensitized MDA-MB-231 cells to I2-induced colony formation inhibition **(Fig. 3B**). Combination of LSD1 inhibitor Tranylcypromine (TCP) and I2 resulted in synergistic effect in blocking invasion of MDA-MB-231 cells (**Fig. 3C**). These findings suggest that inhibition of LSD1 could improve the therapeutic efficacy of PD-1 blockade in breast cancer. We also explored the roles of LSD1 homolog, LSD2 (KDM1B), in breast oncogenesis. We demonstrated that *in vitro* LSD2 overexpression significantly promotes cellular proliferation; and augments colony formation in soft agar; while attenuating motility and invasion (**Fig. 4A-D**). We found that overexpression of LSD2 protein significantly altered expression of key important epigenetic modifiers such as LSD1, HDAC5 (**Fig. 4E**). We also found that LSD2 overexpression in MDA-MB-231 cells facilitated mammosphere formation, enriched CD49f+/EpCAM-subpopulation (**Fig. 4F**). Stable overexpression of LSD2 resulted in accelerated growth of MDA-MB-231 xenograft tumors in nude mice (**Fig. 4G**). These studies provide novel insight into the previously unrecognized roles of LSD2 in human breast cancer cells. We have shown for the first time that LSD2 augments proliferative and cancer stem cell traits,

	and attenuates motility and invasiveness of breast cancer cells. All of these findings suggest that LSD2 has complex and multifaceted roles in breast oncogenesis. In the future, better understanding of epigenetic downstream target genes and pathways controlled by LSD2 would aid in developing novel small molecule inhibitors and combination strategies which might confer selective effects against breast cancer.

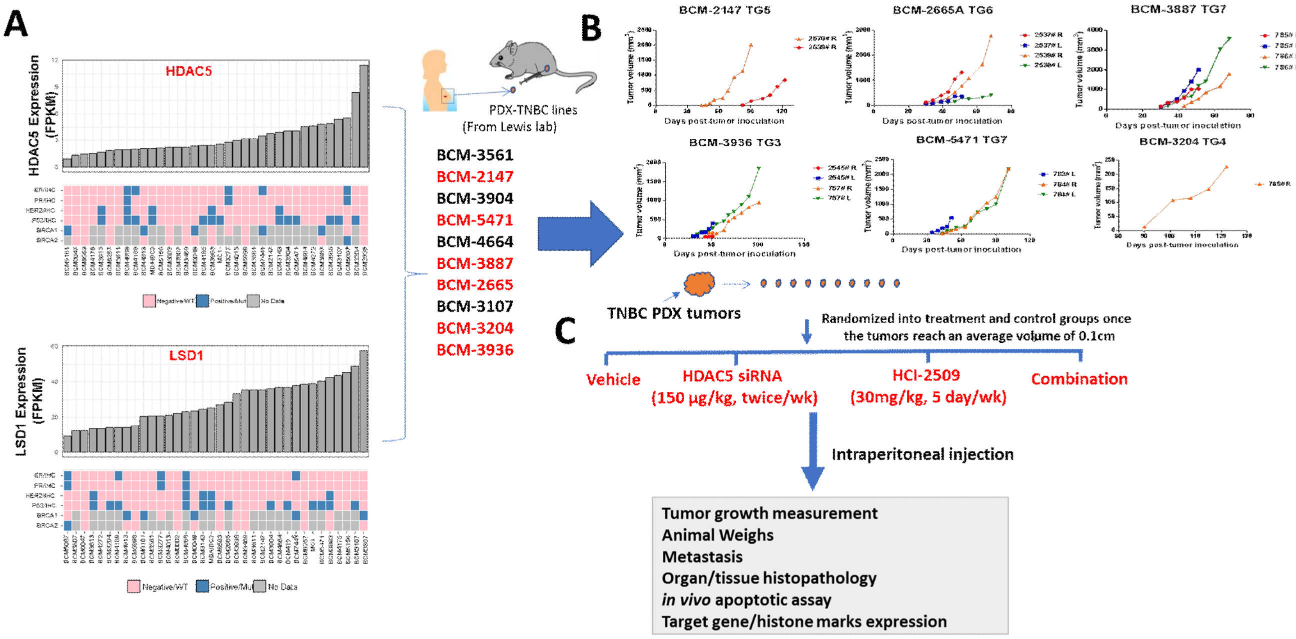

Figure 1: Evaluate therapeutic effects of combination strategies in patient-derived xenografts (PDXs). (A) The expression levels of HDAC5 and LSD1 were examined in a group of TNBC PDX tumor lines using ChIP-seq dataset. 10 TNBC PDX lines expressing relatively higher levels of LSD1 and HDAC5 were selected and retransplanted into mammary glands of immune-compromised SCID/Beige mice. **(B)** Growth of TNBC PDX tumors in SCID/Beige mice. **(C)** Working flow of ongoing studies using PDX model (Aim3).

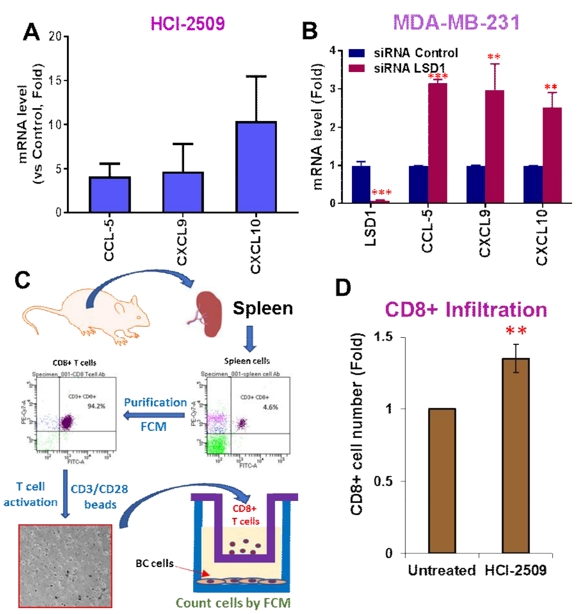

Figure 2. Inhibition of LSD1 induces expression of CD8+ T cell attracting chemokines. (A) LSD1 inhibitor HCI-2509 (2.5 μM) induces mRNA expression of CCL5, CXCL9 and CXCL10. **(B)** Depletion of LSD1 by siRNA in MDA-MB-231 cells increases mRNA levels of CCL5, CXCL9 and CXCL10. (C) *Ex vivo* chemotaxis assay. (D) HCI-2509 enhances CD+8 T cell infiltration to breast tumor cells.

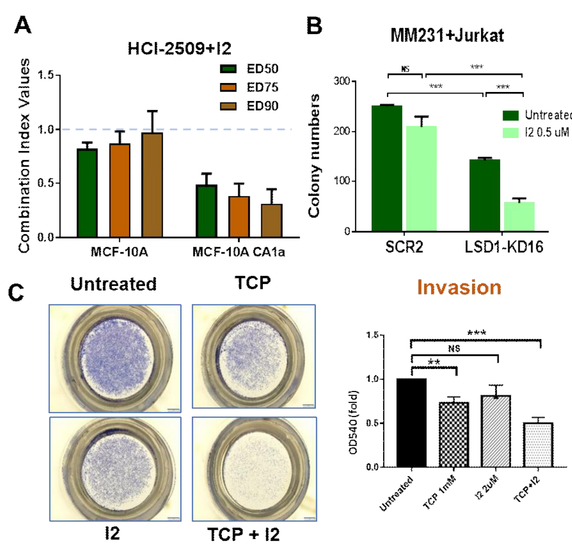

Figure 3. LSD1 inhibitor sensitizes breast cancer cells to PD-1 inhibitor-induced growth and invasion inhibition. (A) Effect of combination therapy on growth of MCF10A and MCF10A-CA1a cells. Synergy was defined as CI < 1, additivity as CI = 1 and antagonism as CI > 1. **(B)** 500 scramble and LSD1-KD MDA-MB-231 cells were plated on 10-cm dishes. After 14 days, colonies formed were stained with crystal violet and counted. **(C)** MDA-MB-231 cells were treated with TCP, I2 alone or simultaneously. A transwell invasion assay was performed to detect the invasive capacity of tumor cells.

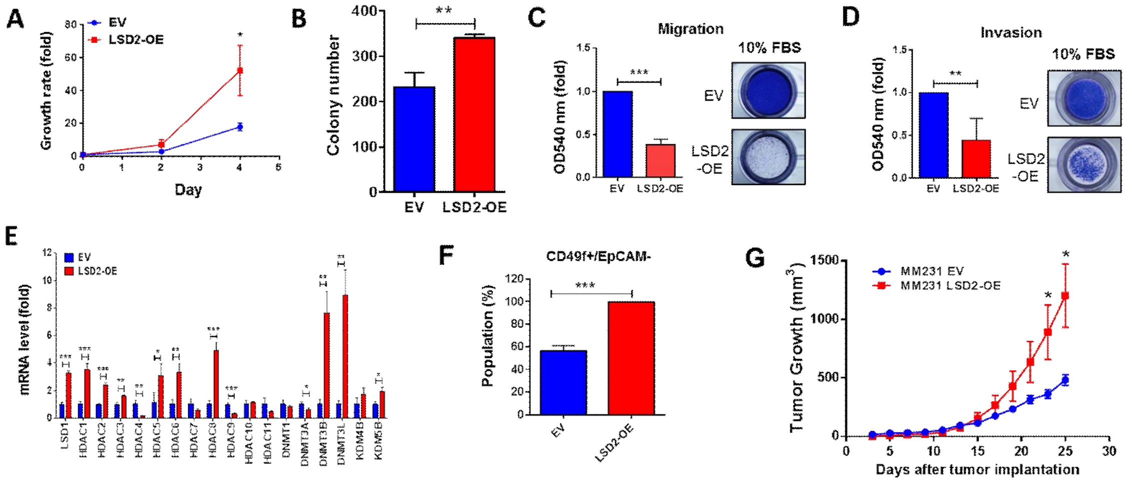

Figure 4. Role of LSD2 in breast cancer oncogenesis. (A) MDA-MB-231 cells were transfected with control empty vector (EV) or LSD2 overexpression vector (LSD2-OE) were analyzed for growth using fluorometric dsDNA quantitation method. **(B)** 500 cells stably transfected with control or LSD2 expression plasmids were plated in 10cm dish. After 14 days, colonies formed were stained with 0.5% crystal violet and counted. **(C)** Transwell migration assay was performed to detect the migratory capacity of MDA-MB-231 EV and LSD2-OE cells. **(D)** Transwell migration assay was performed to detect the invasive capacity of MDA-MB-231 EV and LSD2-OE cells. **(E)** RNA was extracted from MDA-MB-231 EV and LSD2-OE cells and cDNA was synthesized and subjected to quantitative real-time PCR for the indicated gene using TaqMan probes. **(F)** Flow cytometry analysis of cell surface marker CD49f and EpCAM in EV and LSD2-OE cells. **(G)** MDA-MB-231 cells transected with empty vectors (n=17) or LSD2 expression vectors (n=16) were transplanted into the mammary fat pad of nude mice. Tumor volumes were regularly assessed every two days. Shown are average tumor volumes ± s.e.

b. What opportunities for training and professional development has the project provided?

This award has provided an excellent vehicle for postdoctoral fellows and students working on this project to advance their career goal in breast cancer and transition to an independent investigator. One postdoc fellow has completed his postdoc fellow training at UPCI and obtained an independent faculty position at a Chinses university. He will continue to carry out breast cancer research during his faculty tenure. This award also provided excellent research opportunities for UPCI Academy summer student and visiting student from Tsinghua University, China.

c. How were the results disseminated to communities of interest?

We will share data and research results generated from this project with other researchers in accordance with the University of Pittsburgh and NIH Grant Policies on sharing of unique research resources. All model organisms, cell lines, plasmids, pharmacological compounds and reagents generated under this project will be disseminated in accordance with policies of University of Pittsburgh and NIH policies. Depending on such policies, materials may be transferred to others under the terms of a material transfer agreement (MTA). We anticipate that publications of research data will occur during the project. Research data that documents, supports and validates research findings will be made available after the main findings have been accepted for publication.

What do you plan to do during the next reporting period to accomplish the goals?

This project has undergone no-cost extension for an extra year to allow us to complete the investigation on the role of crosstalk between LSD1 and HDAC5 in governing gene expression

and activity that are involved in regulation of breast cancer progress. During the next reporting period, we plan to perform the following studies to accomplish the goals: (1) to further elucidate the mechanism of crosstalk between HDAC5 and LSD1 in breast cancer tumorigenesis; (2) to assist partner PI team to further evaluate therapeutic effects of combination strategies in patient-derived xenografts (PDXs); (3) to study the role of crosstalk between LSD1 and HDAC5 in regulation of breast cancer immune microenvironment.

4. IMPACT

(a) What was the impact on the development of the principal discipline(s) of the project? Triple negative breast cancer (TNBC) is clinically classified based on the absence of the estrogen receptor, the progesterone receptor, or HER2 receptor overexpression. TNBC is more aggressive and distant metastases to lung, liver, brain and bone are more common in TNBC patients than their hormone receptor-positive counterparts. Unlike breast tumors that overexpress hormone receptors or HER2, druggable targets are not available for TNBC. In this project, we have explored a new and underexplored area of epigenetic targeting in breast cancer because breast cancers arise as a consequence of genetic and epigenetic changes. Increasing lines of evidence indicate that epigenetic activity in breast cancer profoundly influences the hallmarks of this disease such as limitless proliferation, increased cell motility, local invasion and metastasis. The study of this project helps to understand the mechanisms underlying the crosstalk between two key epigenetic enzymes, LSD1 and HDAC5, in TNBC because our recent preclinical and human breast cancer studies suggest that these enzymes play a critical role in this subtype of breast cancer. The information generated from this the proposed work has the potential to generate a new targeted therapy strategy for treatment of this devastating disease.

(b) What was the impact on other disciplines? Nothing to Report

(c) What was the impact on technology transfer? Nothing to Report

5. CHANGES/PROBLEMS

(a) Changes in approach and reasons for change

No major changes in approach have been made since the initiation of the award.

(b) **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to Report

(c) **Changes that had a significant impact on expenditures**

Nothing to Report

(d) **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report.

6. PRODUCTS

(a) Publications, conference papers, and presentations

Research Articles:

Cao C, Vasilatos SN, Bhargava R, Fine J, Oesterreich S, Davidson NE, Huang Y. Functional interaction of histone deacetylase 5 (HDAC5) and lysine-specific demethylase 1 (LSD1) promotes breast cancer progression. *Oncogene*, 36(1):133-145, 2017. PMID:27212032 PMCID: PMC5121103

Chen L, Vasilatos SN, Qin Y, Katz T, Cao C, Wu H, Tasdemir N, Levine KM, Oesterreich S, Davidson NE, Huang Y. Functional characterization of lysine-specific demethylase 2 (LSD2/KDM1B) in breast cancer progression. *Oncotarget*, 2017. DOI: 10.18632/oncotarget.19387

Conference Abstract:

Chen L, Vasilatos SN, Qin Y, Cao C, Wu H, Tasdemir N, Katz TA, Oesterreich S, Davidson NE, Huang Y. New insights into the roles of histone lysine-specific demethylase 2 (LSD2) in breast cancer. 2017 AACR Annual meeting.

(b) **Website(s) or other Internet site(s)** Nothing to Report

(c) **Technologies or techniques** Nothing to Report

(d) **Inventions, patent applications, and/or licenses** Nothing to Report

(e) **Other Products** Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a) Individuals who have worked on the project

Name:	**Yi Huang**	**Steffi Oesterreich**	**Ye Qin**	**Shauna Vasilatos**	**Hao Wu**
Project Role:	Initiating-PI	Partnering-PI	Postdoc Fellow	Technician	Visiting scholar
Researcher Identifier (e.g. ORCID ID):	N/A	N/A	N/A	N/A	N/A
Nearest person month worked:	3.6	1.2	12.0	9.0	0.0
Contribution to Project:	Designed and oversaw the studies to define the basic mechanisms and	Oversaw IHC studies and animal experiments, and interpreted	Postdoc fellow. Performed animal study	Performed microarray studies and data analysis	Carried out the experiments to examine the explore

	biological consequences of the functional interplay between HDAC5/LSD1 in breast cancer	the results generated from *in vivo* studies			the molecular mechanisms underlying SFN induced suppression of HDAC5 transcription
Funding Support:	CDMRP Breast Cancer Breakthrough Award, Breast Cancer Research Foundation	CDMRP Breast Cancer Breakthrough Award	CDMRP Breast Cancer Breakthrough Award	CDMRP Breast Cancer Breakthrough Award	China National Fellowship Fund

b) Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

N/A.

c) What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Partnering PI, Dr. Steffi Oesterreich, will submit her annual report separately.

QUAD CHARTS: N/A

9. **APPENDICES**: A copy of publication in *Oncotarget*

Oncogene (2017) 36, 133–145

www.nature.com/onc

ORIGINAL ARTICLE

Functional interaction of histone deacetylase 5 (HDAC5) and lysine-specific demethylase 1 (LSD1) promotes breast cancer progression

C Cao[1,2,3], SN Vasilatos[1,3], R Bhargava[1,3,4], JL Fine[1,4], S Oesterreich[1,2,3], NE Davidson[1,2,3] and Y Huang[1,2,3]

We have previously demonstrated that crosstalk between lysine-specific demethylase 1 (LSD1) and histone deacetylases (HDACs) facilitates breast cancer proliferation. However, the underlying mechanisms are largely unknown. Here, we report that expression of HDAC5 and LSD1 proteins were positively correlated in human breast cancer cell lines and tissue specimens of primary breast tumors. Protein expression of HDAC5 and LSD1 was significantly increased in primary breast cancer specimens in comparison with matched-normal adjacent tissues. Using HDAC5 deletion mutants and co-immunoprecipitation studies, we showed that HDAC5 physically interacted with the LSD1 complex through its domain containing nuclear localization sequence and phosphorylation sites. Although the *in vitro* acetylation assays revealed that HDAC5 decreased LSD1 protein acetylation, small interfering RNA (siRNA)-mediated HDAC5 knockdown did not alter the acetylation level of LSD1 in MDA-MB-231 cells. Overexpression of HDAC5 stabilized LSD1 protein and decreased the nuclear level of H3K4me1/me2 in MDA-MB-231 cells, whereas loss of HDAC5 by siRNA diminished LSD1 protein stability and demethylation activity. We further demonstrated that HDAC5 promoted the protein stability of USP28, a bona fide deubiquitinase of LSD1. Overexpression of USP28 largely reversed HDAC5-KD-induced LSD1 protein degradation, suggesting a role of HDAC5 as a positive regulator of LSD1 through upregulation of USP28 protein. Depletion of HDAC5 by shRNA hindered cellular proliferation, induced G1 cell cycle arrest, and attenuated migration and colony formation of breast cancer cells. A rescue study showed that increased growth of MDA-MB-231 cells by HDAC5 overexpression was reversed by concurrent LSD1 depletion, indicating that tumor-promoting activity of HDAC5 is an LSD1 dependent function. Moreover, overexpression of HDAC5 accelerated cellular proliferation and promoted acridine mutagen ICR191-induced transformation of MCF10A cells. Taken together, these results suggest that HDAC5 is critical in regulating LSD1 protein stability through post-translational modification, and the HDAC5–LSD1 axis has an important role in promoting breast cancer development and progression.

Oncogene (2017) **36,** 133–145; doi:10.1038/onc.2016.186; published online 23 May 2016

INTRODUCTION

Lysine-specific demethylase 1 (LSD1) is the first identified FAD-dependent histone demethylase that has been typically found in association with a transcriptional repressor complex that includes CoREST, HDAC1/2, BHC80 and others.[1–4] A role for elevated expression of LSD1 has been implicated in tumorigenesis in various cancers including breast cancer.[3,5–9] Studies from our and other laboratories consistently showed that inhibition of LSD1 hindered proliferation of breast cancer cells.[6,8,10] Lim *et al.*[6] reported that LSD1 is highly expressed in estrogen receptor-negative breast cancers. A recent study found that LSD1 is significantly over-expressed in high-grade ductal carcinoma *in situ* or invasive ductal carcinoma versus low/intermediate ductal carcinoma *in situ*.[11] These studies point to a tumor-promoting role for LSD1 in breast cancer. We were among the first to report the use of small-molecule compounds and preclinical treatment strategies that have promise to work through this target in cancer.[8,9,12] The development of novel LSD1 inhibitors is progressing rapidly. For example, a new generation of (bis)urea/(bis)thiourea LSD1 inhibitors displayed improved potency against LSD1 in cancer cells.[13] A newly reported GSK-LSD1 inhibitor exhibited interesting cell type-specific inhibition against small-cell lung cancer cells in preclinical models.[14]

However, how LSD1 is upregulated in breast cancer and the precise role of LSD1 in breast cancer development are still unclear. Our most recent work showed that small interfering RNA (siRNA)-mediated inhibition of HDAC5 led to a significant increase of H3K4me2, a known substrate of LSD1, suggesting a potential role of HDAC5 in regulating LSD1 activity.[10] However, little is known about the precise role of HDAC5 and mechanisms underlying its regulation on LSD1 activity in breast cancer. HDAC5 is an important member of class IIa histone deacetylase (HDAC) isozymes with important functions in transcriptional regulation, cell proliferation, cell cycle progression and cellular developmental activities.[15,16] HDAC5 has been shown to have important roles in many diseases including cancer.[17,18] In this study, we addressed the following clinically relevant issues that have been under-studied: (1) Is elevation of LSD1 expression associated with HDAC5 overexpression during breast cancer development? (2) How is LSD1 regulated by HDAC5 in breast cancer? (3) What is the role of the HDAC5–LSD1 axis in breast cancer initiation, proliferation and metastasis? To answer these questions, we delineated the

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mechanisms underlying the functional link between LSD1 and HDAC5 in chromatin remodeling and demonstrated that these two important chromatin modifiers closely cooperate to mediate proliferation, cell cycle and metastasis of breast cancer cells.

RESULTS

HDAC5 and LSD1 proteins are coordinately expressed in human breast cancer

To study the potential association of HDAC5 and LSD1 in breast cancer, we first examined mRNA levels of HDAC5 and LSD1 in human immortalized normal mammary epithelial MCF10A cells, fully malignant MCF10A-CA1a cells transformed from MCF10A cells with transfection of *HRAS*,[19] and several human breast cancer cell lines. Quantitative PCR (qPCR) studies showed that there was no clear association of mRNA expression between HDAC5 and LSD1 in breast cancer cell lines (Figure 1a). The Oncomine-TCGA database showed moderate change of the mRNA level of LSD1 and HDAC5 in IBC (Supplementary Figures 1a and b). mRNA levels of both HDAC5 and LSD1 are altered in ~6% of breast cancer patients (www.cbioportal.org) without an apparent association with specific subtypes (Supplementary Figures 1c and d). However, protein expression of both HDAC5 and LSD1 was significantly elevated in malignant breast cell lines compared with MCF10A (Figure 1b), and protein levels of HDAC5 and LSD1 were positively correlated (Figure 1c). The correlation of HDAC5 and LSD1 protein expression was further validated in 50 primary breast cancers using immunohistochemical staining with validated antibodies (Supplementary Figures 2a and b). The χ^2 analysis showed a statistically significant correlation between HDAC5 and LSD1 protein expression in these tumors (Figure 1d). Furthermore, the immunohistochemistry (IHC) analysis showed that breast cancer tissues ($n=18$) expressed significantly higher level of HDAC5 and LSD1 than matched-normal adjacent tissues ($n=18$) (Figure 1e). The mean H-score for HDAC5 staining in stage 3 breast tumors ($n=25$) was statistically significantly higher than stage 2 counterparts ($n=25$). The mean H-score of LSD1 staining for stage 3 tumors was also higher than that of stage 2 tumors with a *P*-value of 0.07 (Figure 1f). These results were further validated with independent manual H-score evaluations by two breast cancer pathologists with moderate interobserver concordance (Supplementary Figures 3a and b). Taken together, these findings suggest that HDAC5 and LSD1 proteins are coordinately over-expressed in breast cancer cell lines and tissue specimens.

Physical interaction of LSD1 and HDAC5 in breast cancer cells

To address whether LSD1 and HDAC5 physically interact, a co-immunoprecipitation study was carried out in MDA-MB-231 and MCF10A-CA1a cells transiently transfected with pcDNA3.1 or pcDNA3.1-FLAG-HDAC5 plasmids. After immunoprecipitation (IP) with LSD1 antibody, we found that both endogenous and exogenous HDAC5 proteins were co-immunoprecipitated with LSD1 protein (Figure 2a). The interaction between native LSD1 and HDAC5 was further validated in additional breast cancer cell lines (Figure 2b). A similar result was obtained in the reciprocal immunoprecipitation using anti-FLAG antibody to confirm that LSD1 was co-immunoprecipitated with FLAG-HDAC5 (Figure 2c). To precisely map the HDAC5 domain(s) responsible for interaction with LSD1, we expressed a series of HDAC5 deletion mutants engineered in pcDNA3.1-FLAG plasmids in MDA-MB-231 cells (Figure 2d). Immunoprecipitation assays of cells transfected with full-length HDAC5 complimentary DNA (cDNA) confirmed the HDAC5–LSD1 interaction and deletion of an N-terminal myocyte enhancer factor-2 (MEF2) binding domain (HDAC5-Δ1) alone had no impact on HDAC5-LSD1 interaction. However, removal of both the MEF2 domain and nuclear localization sequence (NLS) (HDAC5-Δ2) completely abolished HDAC5–LSD1 interaction. Further deletion of an N-terminal HDAC and nuclear export sequence (HDAC5-Δ3) and MEF2 domain (HDAC5-Δ4) did not adversely alter LSD1 binding with HDAC5 fragments (Figure 2e). Immunofluorescence studies showed nuclear localization of full-length HDAC5, HDAC5-Δ1, HDAC5-Δ3 and HDAC5-Δ4. Depletion of the NLS-containing domain (HDAC5-Δ2) completely blocked HDAC5 nuclear translocation (Figure 2f). *In vitro* pull-down assays by using His-tag recombinant LSD1 protein incubating with HDAC5 full-length or deletion mutants validated that HDAC5 domain containing NLS element is essential for interaction with LSD1 (Supplementary Figure 4).

HDAC5 promotes LSD1 protein stability and activity

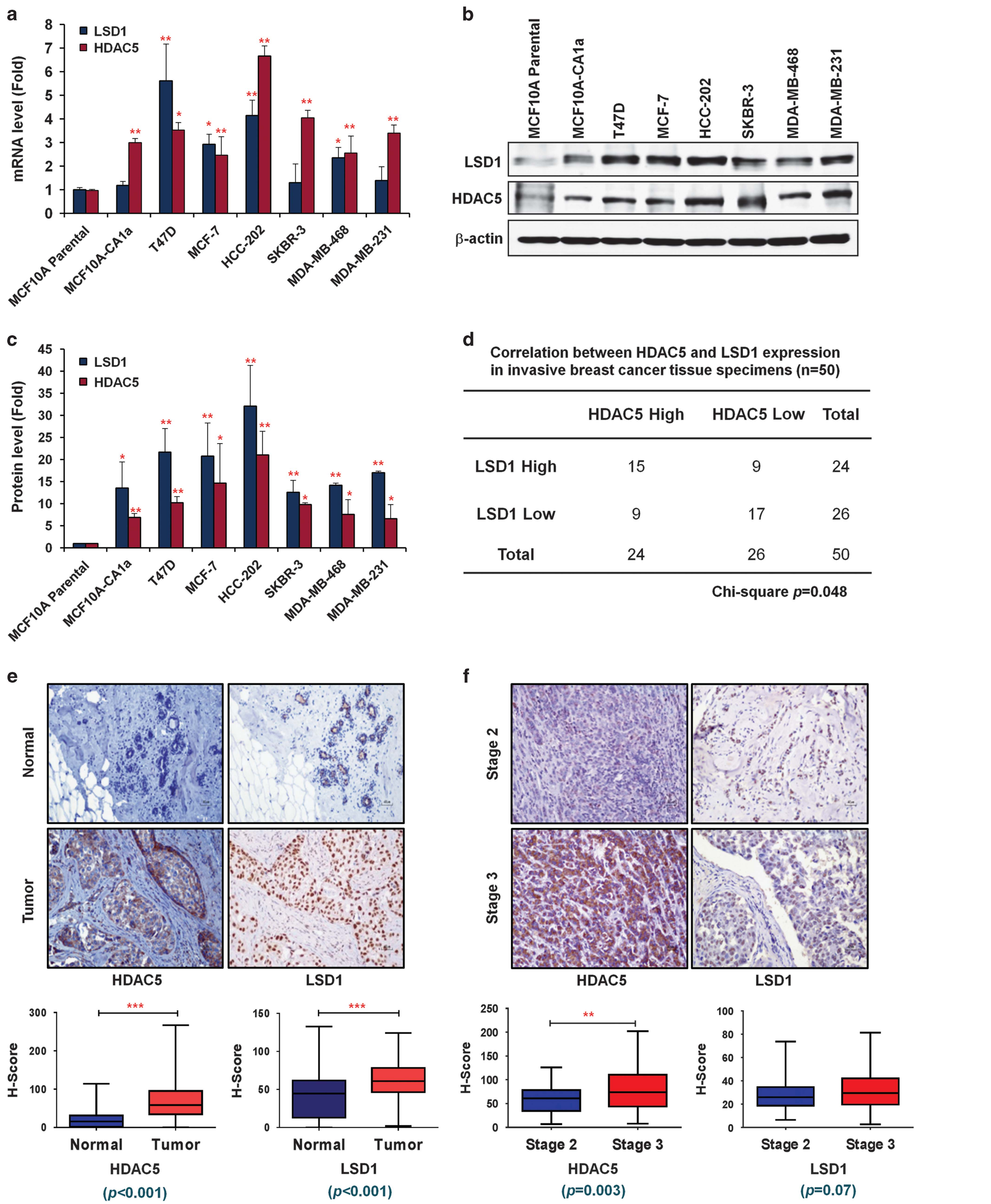
Next, we examined whether the mRNA or protein levels of HDAC5 and LSD1 were affected by their interaction with each other. Overexpression of HDAC5 in MDA-MB-231 cells failed to alter LSD1 mRNA expression, but led to a significant increase of LSD1 protein expression (Figures 3a and b). HDAC5 knockdown by siRNA attenuated LSD1 protein expression without affecting its mRNA level (Figures 3c and d). The effect of LSD1 on HDAC5 expression was subsequently assessed using our previously established MDA-MB-231-LSD1-KD cells.[10] Depletion of LSD1 exerted no effect on HDAC5 mRNA or protein levels (Figures 3e and f). Simultaneous overexpression of pcDNA3.1-HDAC5 with HDAC5 siRNA significantly reversed the decrease of LSD1 (Supplementary Figure 5a). These results suggest that HDAC5 functions as an upstream regulator that governs LSD1 protein stability via post-translational regulation. Quantitative immunoblots showed that levels of H3K4me1/2 and AcH3K9, the substrates for LSD1 and HDAC5, respectively, were downregulated by HDAC5 overexpression, whereas loss of HDAC5 exerted the opposite effect (Figure 3g; Supplementary Figure 5b), suggesting a critical role of HDAC5 in governing chromatin modifying activity of LSD1. The cycloheximide chase assay showed that overexpression of HDAC5 significantly extended LSD1 protein half-life, whereas depletion of HDAC5 by siRNA decreased LSD1 protein half-life in MDA-MB-231 cells (Figures 3h and i; Supplementary Figure 5c). To determine whether other recognized LSD1 cofactors or HDACs exert similar effects on LSD1 protein stability, MDA-MB-231 cells were treated with siRNA against several LSD1 complex cofactors (CoREST, HDAC1 and HDAC2) or other class II HDAC isozymes (HDAC 4, 6, 7, 9, 10), respectively. Transfection with siRNA probes effectively knocked down mRNA expression of target genes without affecting LSD1 protein level (Figure 3j; Supplementary Figure 6a). To confirm the qPCR results, quantitative immunoblotting (IB) was performed and showed depletion of

Figure 1. Correlated overexpression of HDAC5 and LSD1 protein in breast cancer. (**a**) The levels of mRNA expression of HDAC5 and LSD1 in breast cancer cell lines versus MCF10A cells (set as fold 1) using real-time qPCR with β-actin as an internal control. (**b**) Immunoblots with anti-HDAC5 and LSD1 antibodies in indicated cell lines. β-actin protein was blotted as a loading control. (**c**) Histograms represent the mean protein levels of HDAC5 or LSD1 in three determinations relative to β-actin ± s.d. as determined by quantitative immunoblots. (**d**) 50 primary human invasive breast tumor samples were immunostained with antibodies against HDAC5 or LSD1. The χ^2 study was performed by using median H-scores as the cutoff for high- versus low-protein expression. (**e**) Representative HDAC5 and LSD1 staining (200×) in invasive breast carcinoma and adjacent normal tissue specimens from one representative patient. H-scores represent average staining intensity in breast tumors ($n=18$) versus adjacent normal breast tissue ($n=18$). (**f**) Representative HDAC5 and LSD1 staining (200×) in stage 2 and 3 invasive breast carcinoma specimens. H-scores represent average staining intensity in stage 3 breast tumors ($n=25$) versus stage 2 breast tumors ($n=25$). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Student's *t*-test.

CoREST led to insignificant change of LSD1 protein stability (Supplementary Figure 6b and 6c). Together, these results strengthen the conclusion that HDAC5 functions as a positive regulator of LSD1 protein in breast cancer cells.

HDAC5 regulates LSD1 protein stability through modulation of the LSD1-associated ubiquitination system

Protein ubiquitination assays indicated that HDAC5 overexpression significantly attenuated LSD1 polyubiquitination (Figure 4a),



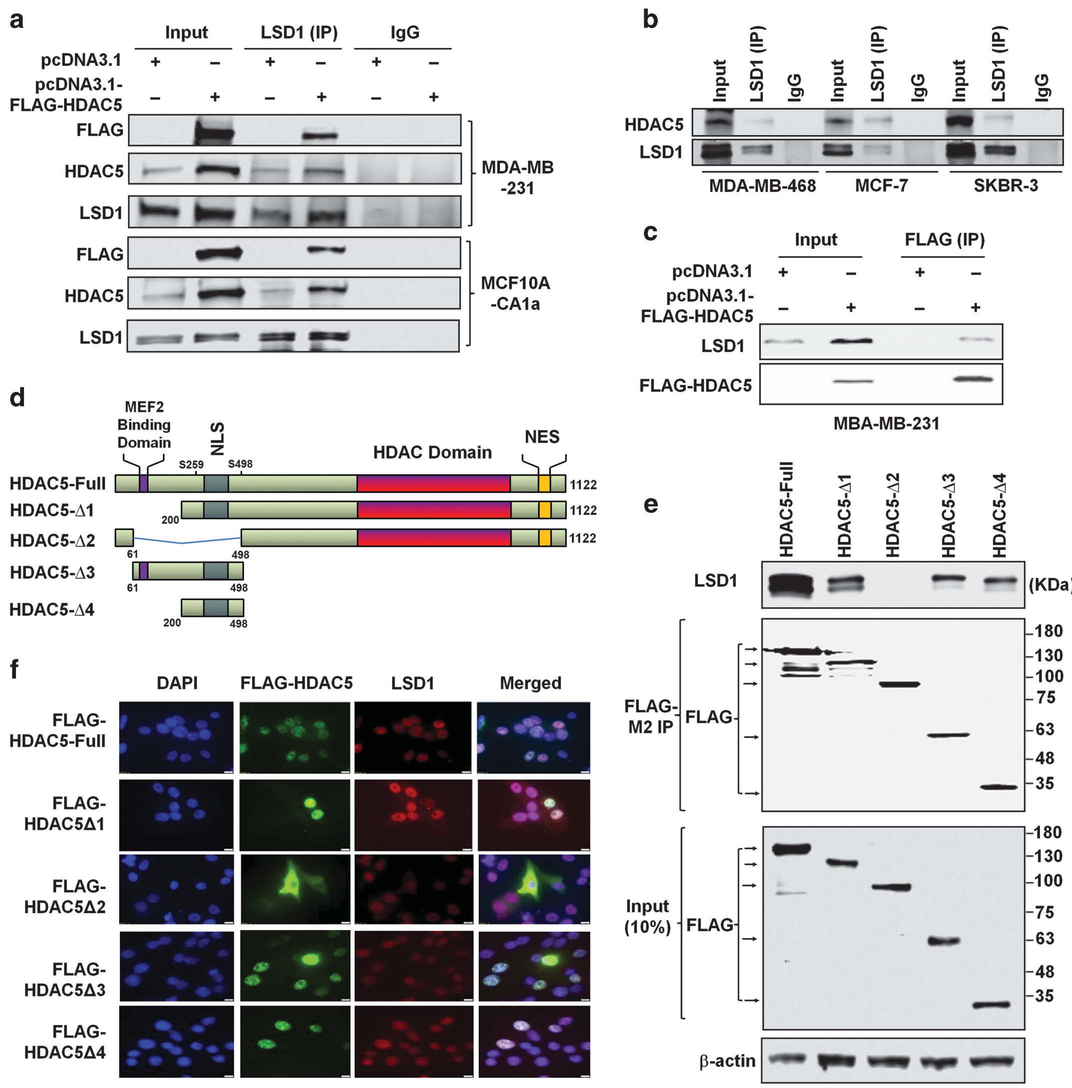


Figure 2. HDAC5 and LSD1 physically interact in breast cancer cells. (**a**) MDA-MB-231 or MCF10A-CA1a cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids. IP was performed with anti-LSD1 antibody followed by immunoblotting (IB) with anti-LSD1, anti-FLAG or anti-HDAC5 antibodies, respectively. (**b**) Whole-cell lysates were immunoprecipitated with anti-LSD1 antibody followed by IB with anti-HDAC5 and LSD1 antibodies in indicated breast cancer cell lines. IgG was used as negative control. (**c**) MDA-MB-231 cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5-FLAG plasmids, and IP was performed with anti-FLAG followed by IB with anti-LSD1 and anti-FLAG antibodies, respectively. (**d**) Schematic representation of full-length and deletion mutants of HDAC5-FLAG constructs. (**e**) FLAG-tagged full-length or deletion mutants of HDAC5 were expressed in MDA-MB-231 cells. Extracts were immunoprecipitated with anti-FLAG antibody, and bound LSD1 was examined by IB using anti-LSD1 antibody. IB with anti-FLAG was used to detect the levels of FLAG-tagged HDAC5 full-length or deletion mutants in IP and input (10%) samples. (**f**) MDA-MB-231 cells were transfected with plasmids expressing FLAG-tagged full-length or deletion mutants of HDAC5 proteins. Immunofluorescence study was performed using anti-FLAG antibody. 4,6-Diamidino-2-phenylindole was used as a control for nuclear staining. All the experiments were performed three times with similar results.

whereas depletion of HDAC5 by siRNA facilitated LSD1 polyubiquitination (Supplementary Figure 7a). Recently, Jade-2 and USP28 were identified as specific E3 ubiquitin ligase and deubiquitinase for LSD1, respectively.[20,21] Our study showing that increase of LSD1 protein expression by Jade-2 siRNA and decrease of LSD1 protein expression by USP28 siRNA in MDA-MB-231 cells confirmed the roles of Jade-2/USP28 as LSD1 ubiquitin ligase/deubiquitinase in breast cancer cells (Figure 4b; Supplementary Figure 7b). qPCR studies demonstrated that mRNA level of either Jade-2 or USP28 was not altered by HDAC5 knockdown or overexpression (Figure 4c). The regulation of HDAC5 on protein expression of Jade-2 or USP28 was subsequently assessed. Due to the lack of highly specific antibody against Jade-2, plasmids expressing Jade-2-FLAG fusion protein were transfected into cells as an alternative approach. MDA-MB-231 and MCF10A-CA1a cells expressing Jade-2-FLAG protein were simultaneously treated with HDAC5 siRNA to evaluate the effect of HDAC5 on Jade-2 protein expression. Immunoblot showed that depletion of HDAC5 did not change the protein level of Jade-2 (Figure 4d). However, overexpression of HDAC5 led to significant increase of USP28 protein expression in both cell lines (Figure 4e). *In vitro* pull-down assay using His-tag recombinant LSD1 protein incubated with USP28-FLAG protein indicated a direct interaction of LSD1 and USP28 (Supplementary Figure 4), and HDAC5 overexpression significantly attenuated USP28 polyubiquitination (Supplementary Figure 7c). To understand whether HDAC5 may stabilize LSD1 protein through upregulation of USP28 protein stability, a rescue study was carried out in MDA-MB-231 and MCF10A-CA1a cells using concurrent transfection of HDAC5 siRNA and USP28 expression plasmids, and showed that overexpression of USP28

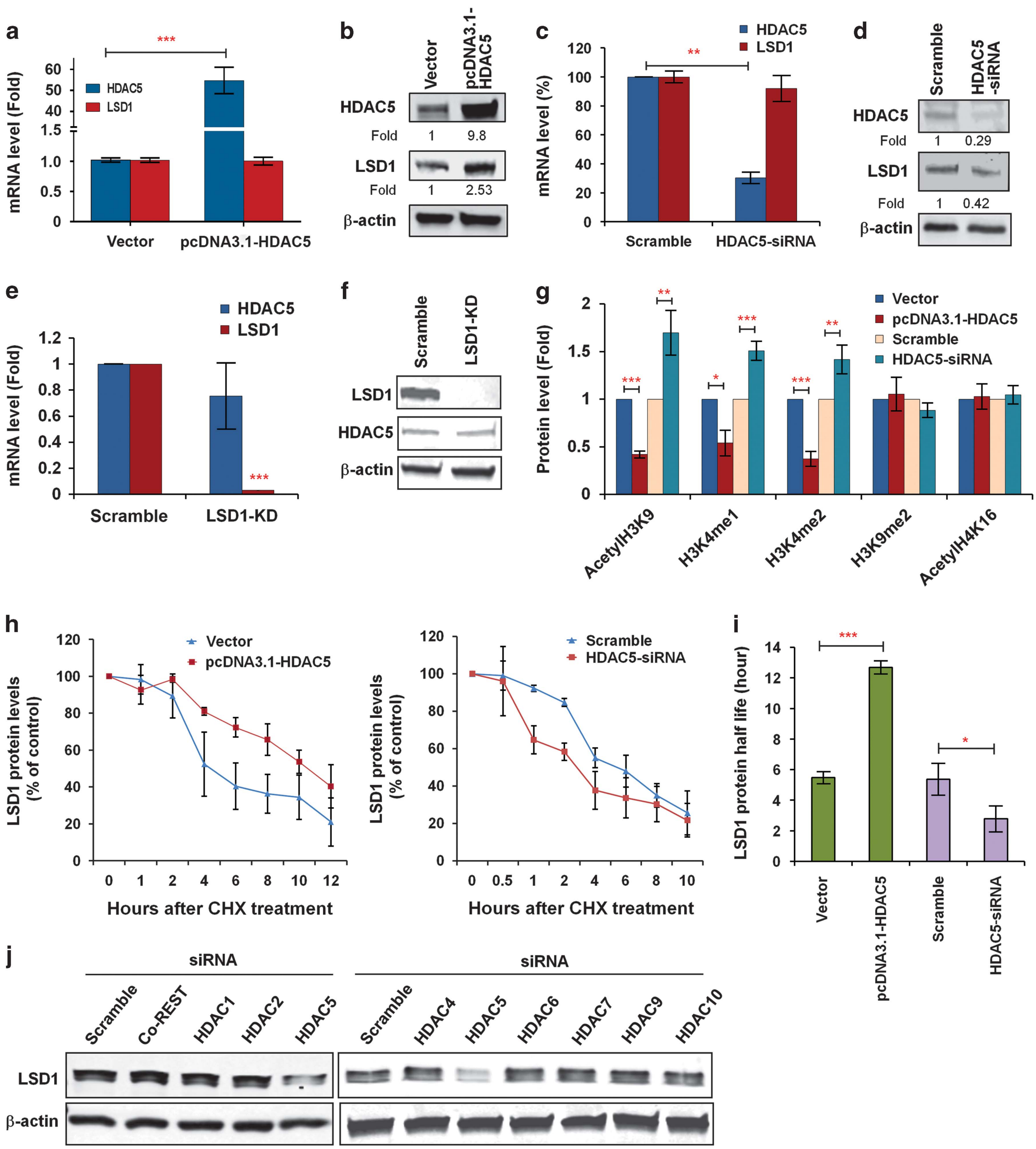


Figure 3. HDAC5 stabilizes LSD1 protein in breast cancer cells. (**a**) MDA-MB-231 cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 for 48 h. mRNA expression of HDAC5 and LSD1 was measured by quantitative real-time PCR with β-actin as an internal control. (**b**) MDA-MB-231 cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids for 48 h. Effect of HDAC5 overexpression on LSD1 protein expression in MDA-MB-231 cells was evaluated by immunoblots with anti-LSD1 and anti-HDAC5 antibodies. (**c**) MDA-MB-231 cells were transfected with scramble siRNA or HDAC5 siRNA for 48 h. Effect of HDAC5 knockdown on LSD1 mRNA expression was examined by quantitative real-time PCR with β-actin as internal control. (**d**) Effect of HDAC5 siRNA on LSD1 protein expression in MDA-MB-231 cells. (**e**) Effect of depletion of LSD1 on mRNA expression of HDAC5 in MDA-MB-231-Scramble or MDA-MB-231-LSD1-KD cells. (**f**) Effect of LSD1-KD on protein expression of HDAC5 in MDA-MB-231-scramble or MDA-MB-231-LSD1-KD cells. (**g**) MDA-MB-231 cells were transfected with control vector pcDNA3.1, pcDNA3.1-HDAC5, scramble siRNA or HDAC5 siRNA for 48 h and analyzed by immunoblots for nuclear expression of indicated histone marks. Proliferating cell nuclear antigen was used as loading control. (**h**) Effect of HDAC5 overexpression or siRNA on LSD1 protein half-life in cycloheximide chase study. (**i**) Measurement of LSD1 half-life using the Calcusyn program. (**j**) Effect of siRNA knockdown of LSD1 cofactors or class II HDACs on LSD1 protein level. All the experiments were performed three times. Bars represent the mean of three independent experiments ± s.d. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test.

completely blocked the destabilization of LSD1 by HDAC5 depletion (Figure 4f; Supplementary Figure 7d). In an additional rescue experiment, overexpression of HDAC5 failed to promote LSD1 protein expression when cells were simultaneously treated with USP28 by siRNA (Supplementary Figure 7e). All these data support the notion that HDAC5 stabilizes LSD1 protein by enhancing protein expression of its deubiquitinase.

To examine whether interaction of HDAC5 with the LSD1/USP28 complex deacetylates LSD1 or USP28, *in vitro* protein acetylation assays was first carried out by incubating GST-tagged recombinant

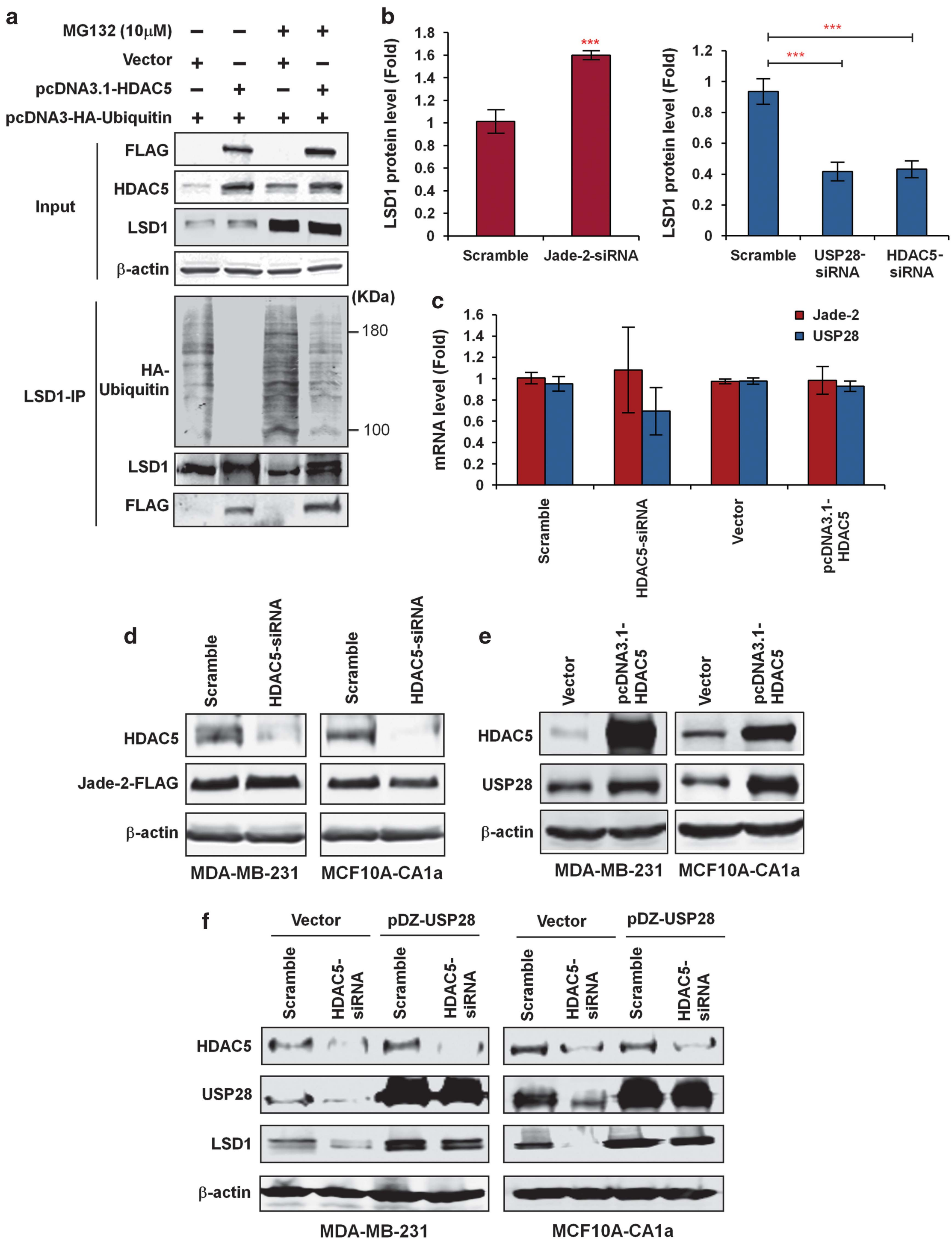


Figure 4. HDAC5 regulates LSD1 by altering USP28 stability. (**a**) MDA-MB-231 cells transfected with pcDNA3.1-FLAG, pcDNA3.1-FLAG-HDAC5 or pcDNA3-HA-ubiquitin plasmids were treated with or without proteasome inhibitor 10 μM MG132 for 10 h followed by IP using LSD1 antibody and immunoblots with anti-HA, LSD1 or HDAC5 antibodies. (**b**) Effect of siRNA of Jade-2, USP28 and HDAC5 on LSD1 protein expression in MDA-MB-231 cells. Results represent the mean of three independent experiments ± s.d. ***$P < 0.001$, Student's *t*-test. (**c**) MDA-MB-231 cells were transfected with scramble siRNA, HDAC5 siRNA, control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids for 48 h. mRNA expression of Jade-2 and USP28 was measured by qPCR. β-actin was used as an internal control. (**d**) MDA-MB-231 or MCF10A-CA1a cells were simultaneously transfected with pcDNA3.1-FLAG-Jade-2 and HDAC5 siRNA for 48 h and subjected to immunoblots with anti-HDAC5 or Jade-2 antibodies. β-actin was used as loading control to normalize target protein levels. (**e**) After MDA-MB-231 or MCF10A-CA1a cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids for 48 h, IB was performed for expression of HDAC5 and USP28. (**f**) MDA-MB-231 or MCF10A-CA1a cells were transfected with scramble or HDAC5 siRNA alone, or in combination with pDZ-USP28 for 48 h. Whole-cell lysates were analyzed for protein levels of HDAC5, USP28 and LSD1. β-actin was used as loading control to normalize target protein levels. The experiments were performed three times with similar results.

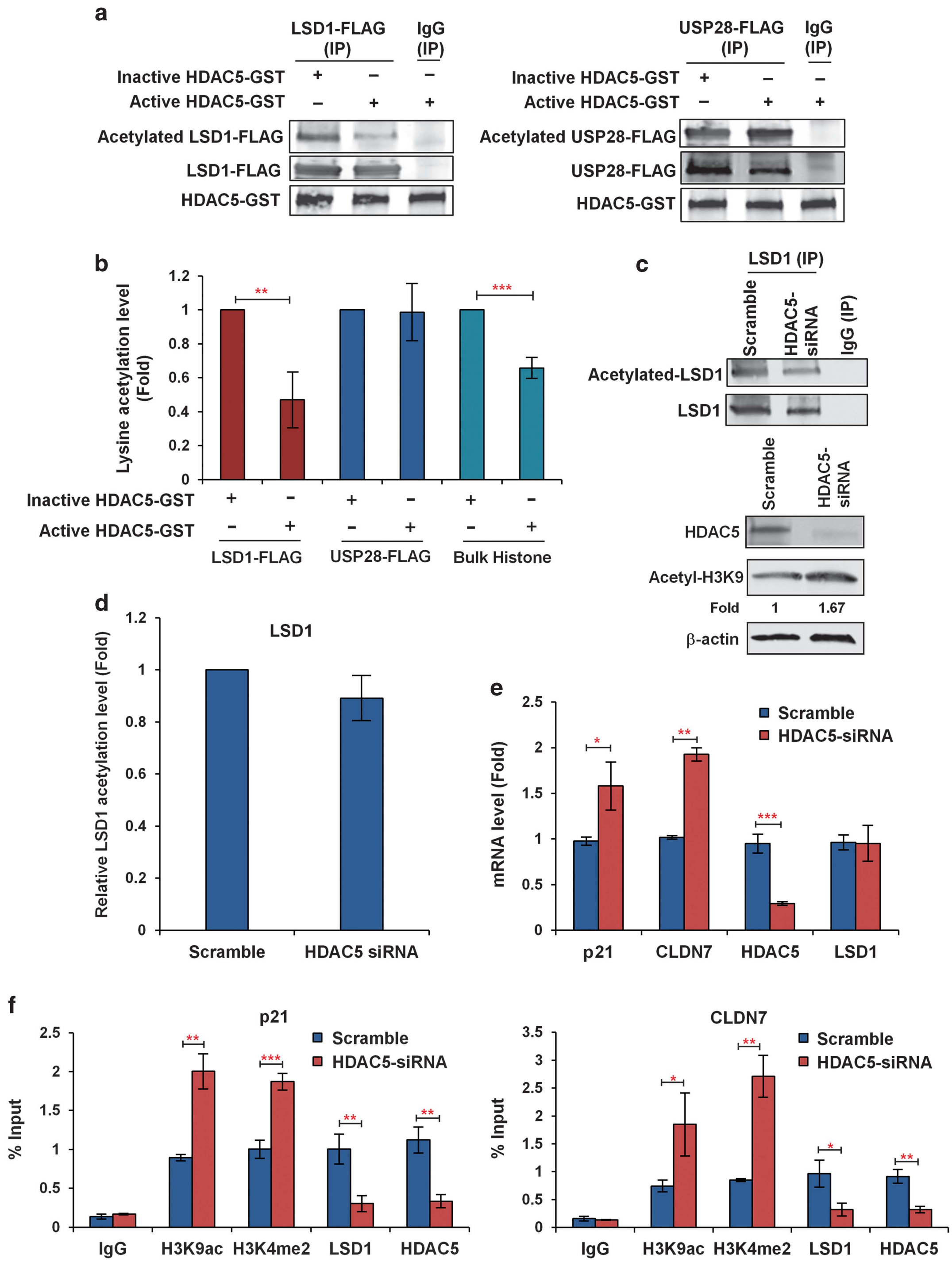
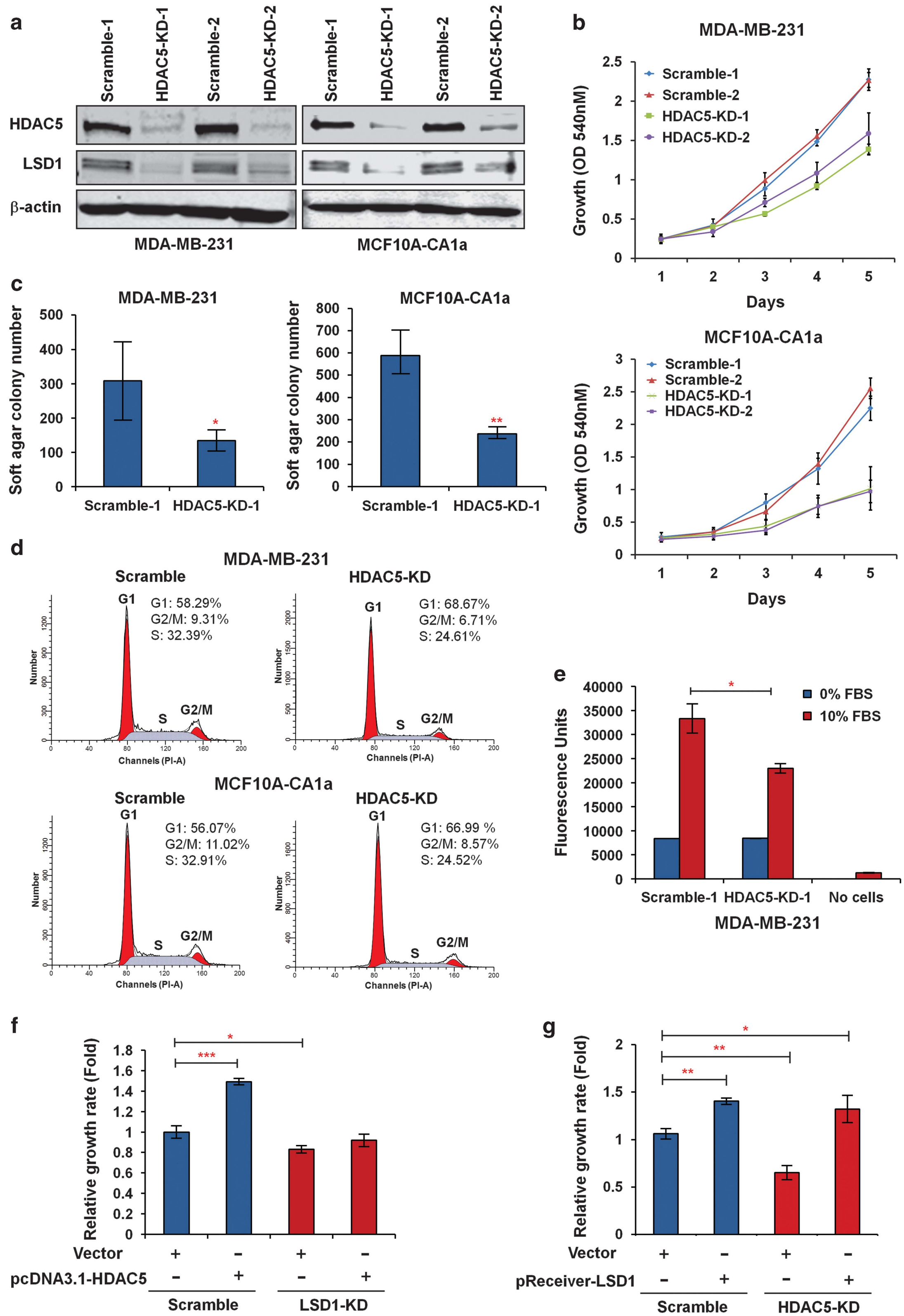


Figure 5. Effect of HDAC5 on protein acetylation of LSD1/USP28 and transcription of LSD1 target genes. (**a**) The immunoprecipitates of FLAG using FLAG-M2 agarose from MDA-MB-231 cells overexpressing FLAG-tagged USP28 or FLAG-tagged LSD1 were used as substrates for protein deacetylation assay. IgG was used as negative control. Active or heat inactivated recombinant human GST-tagged HDAC5 protein were mixed with immunoprecipitates and incubated at 37 °C for 6 h as described in 'Materials and Methods'. The reactions were then subjected to immunoblots with anti-acetyl lysine antibody. FLAG-tagged USP28 or LSD1 proteins were probed with anti-FLAG antibody. HDAC5-GST protein was probed with anti-HDAC5 antibody. (**b**) Histograms represent the means of levels of acetyl-LSD1, acetyl-USP28 and acetyl-histone determined by quantitative IB using infrared IB detection and analysis. (**c**) MDA-MB-231 cell transfected with scramble or HDAC5 siRNAs for 48 h. LSD1 or IgG antibodies were added to cell lysate. IP was performed with anti-LSD1 antibody followed by IB with anti-acetyl lysine and anti-LSD1 antibodies, respectively. Effect of HDAC5 siRNA on AcetylH3K9 protein expression in MDA-MB-231 cells was examined by IB with anti-acetyl-H3K9 antibody. (**d**) Histograms represent the means of relative levels of acetyl-LSD1 determined by quantitative IB using infrared IB detection and analysis. (**e**) mRNA expression of indicated genes in MDA-MB-231 cells transfected with scramble siRNA or HDAC5 siRNA. Data are means ± s.d. of three independent experiments. (**f**) Quantitative chromatin immunoprecipitation (ChIP) analysis was used to determine the occupancy by acetyl-H3K9, H3K4me2, LSD1 and HDAC5 at promoters of p21 or CLDN7 in MDA-MB-231 cells transfected with scramble or HDAC5 siRNA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's t-test.

HDAC5 protein with cellular pull-down of LSD1-FLAG or USP28-FLAG by IP, and immunoprecipitates of IgG was incubated with recombinant HDAC5 protein as negative control of assays (Figure 5a). Bulk histone was used as control substrate (Supplementary Figure 8). Quantitative immunoblots using antibody against pan-acetylated lysine showed that HDAC5 reduced acetylation level of LSD1 without altering the acetylation status of USP28 (Figures 5a and b). Next, the *in vivo* effect of HDAC5 depletion on LSD1 acetylation was investigated in MDA-MB-231 cells transfected with scramble or HDAC5 siRNAs. After immunoprecipitation with LSD1 antibody or IgG (negative control), IB was performed and the results showed that expression levels of both



total LSD1 protein and acetylated LSD1 protein were decreased by HDAC5 depletion (Figure 5c). Quantitative immunoblots indicated that the relative acetylation level of LSD1 was not statistically altered by HDAC5 siRNA in MDA-MB-231 cells (Figure 5d). Acetyl-H3K9 was used as control of substrate and its expression was increased by HDAC5 siRNA (Figure 5c). These results suggest that inhibition of HDAC5 alone is not sufficient enough to increase LSD1 acetylation in breast cancer cells.

Inhibition of HDAC5 reactivates expression of LSD1 target genes

In cancer cells, amplified LSD1 expression is frequently associated with abnormal suppression of key tumor suppressor genes.[3,22] We next examined whether expression of LSD1 target tumor suppressor genes could be reactivated following HDAC5 inhibition. Loss of expression of cyclin-dependent kinase inhibitor p21 and epithelial marker claudin-7 (CLDN7) has been reported to be associated with an aggressive phenotype of breast cancer.[23,24] The transcription activity of p21 and CLDN7 has been found to be suppressed by enhanced activity of LSD1 in breast cancer.[6,25] Transfection of HDAC5 siRNA resulted in significantly increased mRNA expression of p21 and CLDN7 in MDA-MB-231 cells (Figure 5e). Quantitative chromatin immunoprecipitation assays revealed that depletion of HDAC5 decreased occupancy of both HDAC5 and LSD1, and increased enrichment of H3K4me2 and acetyl-H3K9 at the promoters of both genes (Figure 5f). These data suggest that transcriptional de-repression of these genes lies largely in the cooperation between HDAC5 and LSD1 at key active histone marks.

Inhibition of HDAC5–LSD1 axis hinders breast cancer proliferation and invasion

To explore the functional role of the HDAC5–LSD1 axis in regulating breast cancer development, stable knockdown of HDAC5 mRNA (HDAC5-KD) was generated in MDA-MB-231 and MCF10A-CA1a cells by infection with short hairpin RNA (shRNA) lentiviral particles. Similar to the effect of transient inhibition of HDAC5 by siRNA, stable knockdown of HDAC5 expression significantly reduced LSD1 protein expression in two independent HDAC5-KD clones (Figure 6a). Loss of HDAC5 in both clones hindered cell proliferation and colony formation in soft agar (Figures 6b and c). The flow cytometry analysis showed that inhibition of HDAC5 resulted in a greater fraction of cells accumulated at G1 phase and reduction of the S-phase cell fraction (Figure 6d; Supplementary Figure 9). Moreover, loss of HDAC5 attenuated motility and invasion of MDA-MB-231 cells in a Boyden chamber assay (Figure 6e). A rescue experiment indicated that HDAC5 overexpression promoted growth of MDA-MB-231-Scramble cells, but failed to alter the growth of MDA-MB-231-LSD1-KD cells (Figure 6f). An additional rescue study revealed that LSD1 overexpression rescued growth inhibition by HDAC5 depletion in MDA-MB-231-HDAC5-KD cells (Figure 6g). Taken together, these results demonstrate that tumor-promoting activity of HDAC5 is dependent on LSD1 activity in breast cancer cells.

Overexpression of HDAC5 promotes mutagen-induced tumorigenic development in MCF10A cells

To address whether enhanced interaction between HDAC5 and LSD1 is a critical epigenetic alteration driving tumorigenic transformation of breast cancer, we generated two MCF10A cell lines overexpressing HDAC5 (MCF10A-HDAC5). Stable overexpression of HDAC5 in MCF10A cells increased LSD1 protein level and promoted cell proliferation of both clones (Figures 7a and b), indicating a growth-promoting role for HDAC5 in MCF10A cells. Inhibition of LSD1 by shRNA significantly hindered MCF10A growth and reversed the growth promotion mediated by HDAC5 overexpression, suggesting that HDAC5 promotes MCF10A growth in an LSD1 dependent manner (Figure 7c; Supplementary Figure 10). To evaluate if MCF10A-HDAC5 cells have altered susceptibility to tumorigenesis, MCF10A-Vector and MCF10A-HDAC5 cells were cultured for 7 months in medium containing 500 ng/ml ICR191. ICR191 generates genomic instability and genetic variability, and has been successfully used to induce epithelial cell transformation in several models including MCF10A.[26,27] MCF10A-HDAC5 cells were subsequently tested for the capacity of anchorage-independent growth in soft agar for 4 weeks. The soft agar colony formation study demonstrated that ICR191 treatment improved the ability of MCF10A cells to form growing colonies, and overexpression of HDAC5 significantly promoted ICR191-induced colony formation in MCF10A cells (Figure 7d). To determine the role of LSD1 in HDAC5 enhanced tumorigenic transformation induced by ICR191, scramble control and LSD1 shRNA lentivirus particles were infected into MCF10A-Vector or MCD10A-HDAC5 cells, which had been treated with ICR191 for 7 months, and the soft agar growth assays showed that loss of LSD1 in MCF10A-HDAC5 cells significantly abolished cellular ability in colony formation (Figure 7e). A model illustrating the role of HDAC5–LSD1 axis in breast cancer development is proposed based on the above findings (Figure 7f).

DISCUSSION

High levels of HDAC5 have been found to be associated with poor survival in multiple cancer types.[28,29] LSD1 overexpression has been reported to be a poor prognostic factor in basal-like breast cancer, a subtype with aggressive clinical characteristics.[6,30] In this study, the IHC analysis showed that breast cancers expressed higher levels of HDAC5 compared to the matched-normal adjacent breast tissue. Importantly, our study found a positive correlation between HDAC5 and LSD1 proteins in breast tumor cell lines and patient tissue specimens. Increased expression of HDAC5 and LSD1 is correlated with higher stage of breast cancer in our exploratory study. These findings suggest that the coordinated overexpression of HDAC5 and LSD1 may serve as potential novel prognostic markers as well as possible therapeutic targets for breast cancer. More robust studies will be necessary to understand the precise role of elevated protein expression levels of HDAC5 and LSD1 in the risk stratification of breast cancer patients.

Figure 6. HDAC5–LSD1 axis is implicated in breast cancer progression. (**a**) Depletion of HDAC5 by shRNA lentivirus infection downregulated LSD1 protein expression in MDA-MB-231 and MCF10A-CA1a cells. (**b**) Scramble shRNA and HDAC5-KD cells were analyzed for growth and viability by crystal violet assays. (**c**) Soft agar colony formation for HDAC5-KD and scramble control of MDA-MB-231 and MCF10A-CA1a cells. (**d**) Scramble shRNA and HDAC5-KD cells were harvested and stained for DNA with propidium iodide for the flow cytometric analysis. The fractions corresponding to G1, S and G2/M phases of the cell cycle are indicated. (**e**) The Boyden Chamber transwell migration assays for cell invasion for MDA-MB-231-Scramble or MDA-MB-231-HDAC5-KD-1 cells. (**f**) MDA-MB-231-Scramble or MDA-MB-231-LSD1-KD cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 for 5 days and crystal violet assays for growth were carried out. (**g**) MDA-MB-231-Scramble or MDA-MB-231-HDAC5-KD cells were transfected with empty or pReceiver-LSD1 expression plasmids for 5 days and crystal violet assays for growth were carried out. Bars represent the means of three independent experiments ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test.

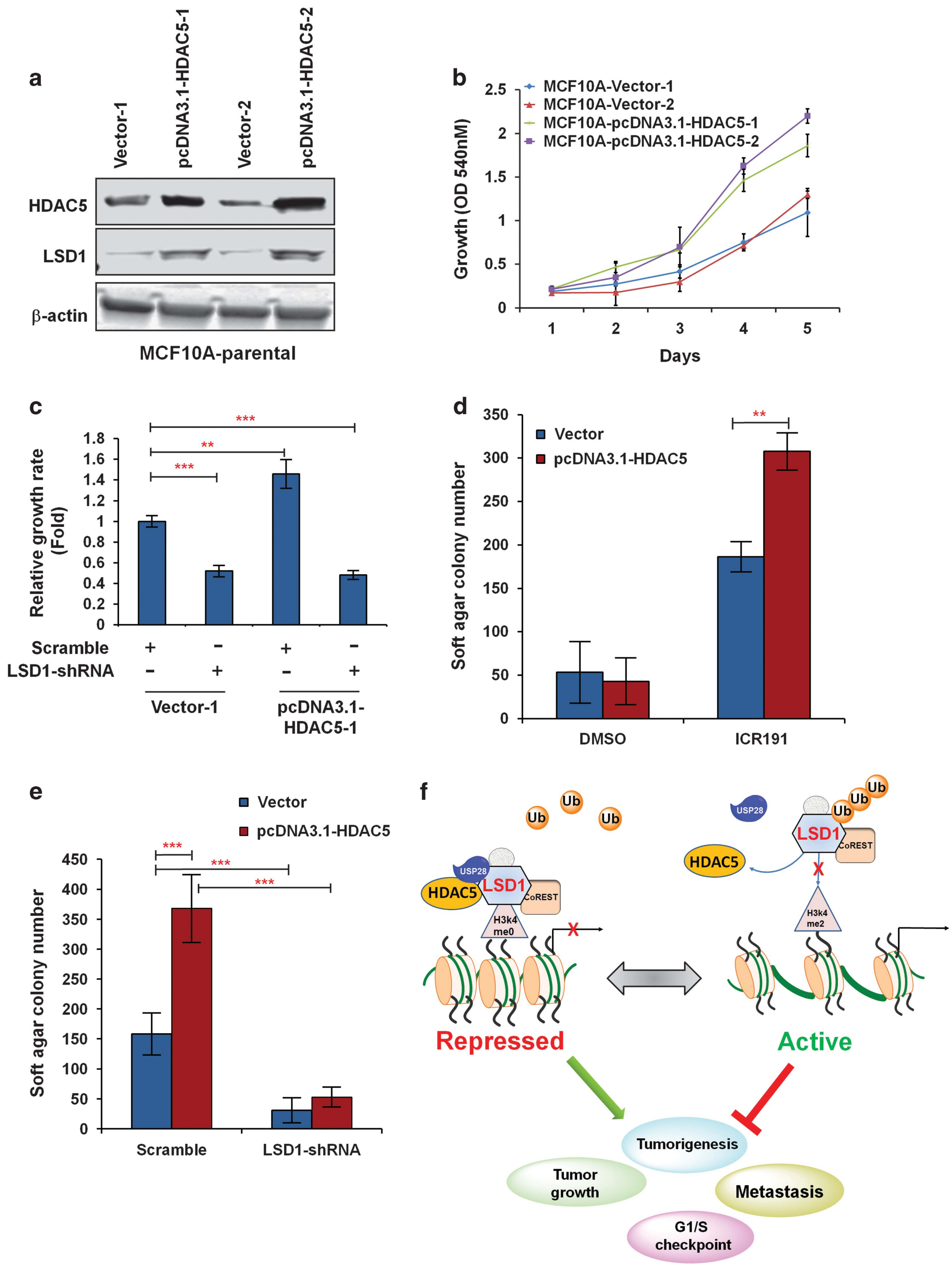


Figure 7. Effect of HDAC5 on growth and mutagen-induced tumorigenic transformation in MCF10A cells. (**a**) pcDNA3.1 or pcDNA3.1-HDAC5 transfected MCF10A cells (clone 1 and 2) were analyzed for protein levels of HDAC5 and LSD1 by immunoblots with anti-HDAC5 and anti-LSD1 antibodies. (**b**) The crystal violet assay for growth of MCF10A stably transfected with control vector or pcDNA3.1-HDAC5 plasmids. (**c**) MCF10A-Vector-1 or MCF10A-HDAC5-1 cells were infected with scramble or LSD1 shRNA lentivirus particles for 5 days followed by crystal violet assays for growth. (**d**) MCF10A cells transfected with pcDNA3.1 or pcDNA3.1-HDAC5 plasmids were treated with dimethyl sulfoxide or 500 ng/ml ICR191 for 7 months followed by soft agar colony formation assays. (**e**) After treatment with 500 ng/ml ICR191 for 7 months, MCF10A-HDAC5 cells were infected with scramble control or LSD1 shRNA lentivirus particles and soft agar colony formation assay was carried out. (**f**) Proposed model of the role of HDAC5–LSD1 axis in breast cancer development. Bars represent the means of three independent experiments ± s.d. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test.

LSD1 protein stability is controlled by several post-translational modifications such as ubiquitination and methylation.[20,21,31] However, the precise mechanism of how LSD1 protein stability is regulated is still not understood. A previous study reported that stable depletion of CoREST facilitated LSD1 degradation in HeLa cells.[32] However, siRNA-mediated knockdown of CoREST alone in breast cancer cells failed to destabilize LSD1 protein, suggesting additional layers of control of LSD1 protein stability are required in breast cancer. In this study, we observed for the first time that LSD1 protein stability is promoted by HDAC5. We further found

that the HDAC5 domain containing NLS is essential for LSD1–HDAC5 interaction. The NLS element provides docking sites for 14-3-3 chaperone binding and has been shown to be critical for HDAC5 import into the nucleus and the regulation of its repressor activity.[17,33] Although an *in vitro* assay demonstrated that HDAC5 reduced LSD1 acetylation, HDAC5 siRNA treatment in breast cancer cells failed to alter acetylation of LSD1 protein. Our *in vivo* results suggest that LSD1 acetylation is likely regulated by a large complex that may involve additional protein deacetylases or cofactors. Further studies are needed to identify the regulatory complex and clarify the precise role of HDAC5 in regulation of LSD1 acetylation in breast cancer cells.

Our studies revealed that HDAC5 regulates LSD1 via enhancement of the protein stability of deubiquitinase USP28. High expression of USP28 has been found to promote the progression of breast and colon cancers.[20,34] Importantly, USP28 has been reported to deubiquitinate important tumor growth regulators such as c-Myc and TP53BP1 that are involved in MYC proto-oncogene stability and DNA damage response checkpoint regulation, respectively.[35,36] Our pilot microarray study revealed that inhibition of the HDAC5–LSD1 axis down-regulates c-Myc expression (data not shown). Sen *et al*.[37] recently reported that HDAC5 is a key component in the temporal regulation of p53-mediated transactivation. All of these findings imply an interaction of HDAC5/LSD1 axis and USP28-associated ubiquitin–proteasome system in regulating downstream targets involved in tumor development. USP28 has been well-characterized for its role in promoting tumorigenesis, and thus is a potential candidate target in cancer therapy. Given the current inability to use drugs to directly target USP28-driven cancer proliferation, our study suggests a novel alternative approach of targeting USP28 stability by development of HDAC5-specific inhibitors in cancer.

Our findings provide supportive evidence showing that HDAC5 control of cell proliferation is largely dependent on LSD1 stabilization. Furthermore, in this study, we showed that non-transformed MCF10A cells overexpressing HDAC5 significantly promoted ICR191-induced transformation of MCF10A cells. The overexpressed HDAC5 is consistently associated with upregulated LSD1 protein expression over the entire course of transformation induction. These data indicate that enhanced crosstalk between HDAC5 and LSD1 may represent a critical mechanism contributing to breast tumorigenesis. HDAC inhibitors hold great promise for cancer therapy. Despite the promising clinical results produced by the HDAC inhibitors in treatment of hematological cancers such as T-cell lymphoma, no apparent clinical evidence indicates that HDAC inhibitors work effectively as a monotherapy against solid tumors including breast tumors.[38–41] From a clinical perspective, our novel findings have significance for design and development of novel combination strategies targeting HDAC5–LSD1 axis as an alternative approach for improvement of therapeutic efficacy of HDAC inhibitors in breast cancer.

As summarized in Figure 7f, we show for the first time that LSD1 protein stability is promoted by HDAC5 through the LSD1 associated ubiquitin–proteasome system, confirming that the regulation of LSD1 by HDAC5 is a post-translational event. Our novel findings also provide supportive evidence that an orchestrated interaction between HDAC5 and LSD1 is a critical epigenetic mechanism to suppress transcriptional activities of important tumor suppressor genes that may contribute to breast cancer development.

MATERIALS AND METHODS

Reagents and cell culture conditions

MDA-MB-231, MDA-MB-468, MCF-7, T47D, HCC-202 and SK-BR-3 cell lines were obtained from the ATCC/NCI Breast Cancer SPORE program. MCF10A-parental and MCF10A-CA1a cells were gifts from Dr Saraswati Sukumar (Johns Hopkins University). Cells were cultured in growth medium as described previously.[10,42]

Tissue microarrays and immunohistochemistry

Tissue microarrays (US Biomax, Rockville, MD, USA) were stained using LSD1 or HDAC5 antibodies. Standard staining procedure for paraffin sections was used for IHC according to manufacturer's recommendations (Vector Labs Inc., Burlingame, CA, USA). Monoclonal antibodies were used for detection of LSD1 (1:800; Cell Signaling, Danvers, MA, USA) and HDAC5 (1:100; Santa Cruz, CA, USA). The staining was visualized using diaminobenzidine, and quantitated using IHC Profiler, an ImageJ plugin (National Institutes of Health, Bethesda, MD, USA).[43] H-scores were calculated as previously described.[44] The manual scoring of H-scores was also carried out by two breast cancer pathologists.

Plasmid construction and stable transfection

Plasmids pcDNA3.1(+)-FLAG, pcDNA3.1(+)-FLAG-HDAC5 and pDZ-FLAG-USP28 were purchased from Addgene (Cambridge, MA, USA). pReceiver-FLAG-LSD1 was obtained from Gene Copoeia (Rockville, MD, USA). A FLAG-tagged ORF cDNA clone for Jade-2 was purchased from GenScript (Piscataway, NJ, USA). pcDNA3-HA-ubiquitin was obtained from Dr Yong Wan (University of Pittsburgh). HDAC5 deletion mutants were engineered into pcDNA3.1(+)-FLAG-HDAC5 by PCR with primers shown in Table S1. HDAC5-Δ2 was constructed by digesting full-length plasmids with SacII from amino acid 61 to 489. Stable transfection was carried out using Lipofectamine 3000 transfection reagent (Life Technologies, Grand Island, NY, USA), and colonies were selected with 800 μg/ml G418.

siRNA and shRNA treatment and stable cell line generation

Pre-designed siRNA and non-targeting scramble siRNA (Santa Cruz) were transfected into cells following the manufacturer's protocol. Cells were collected 48 h post-transfection for further analysis. Scramble control, LSD1-specific or HDAC5-specific shRNA lentiviral particles (Santa Cruz) were infected into cells according to manufacturer's protocol. Cells were treated with 10 μg/ml puromycin 72 h after infection. Single colonies were analyzed for expression of LSD1 or HDAC5 via immunoblots.

RNA extraction and qPCR

Total RNA extraction and cDNA synthesis used the methods described previously.[10] Quantitative real-time PCR was performed on the StepOne real-time PCR system (Life Technologies). All of the TaqMan gene expression assays were pre-designed and obtained from Life Technologies.

Western blotting

Western blotting was performed as previously described.[12,45,46] Antibodies used in this study were shown in Supplementary Table S2. Membranes were scanned with Li-Cor BioScience Odyssey Infrared Imaging System (Lincoln, NE, USA).

Crystal violet and cell invasion assays

The crystal violet proliferation assays were performed as described in our previous study.[47] The invasive capability of breast carcinoma cells was tested with Millipore QCM 24-well invasion assay kit (Merck KGaA, Germany) according to manufacturer's protocol.

Soft agar colony formation assay

A total of 1.2% Bacto-agar (BD Biosciences, Franklin Lakes, NJ, USA) was autoclaved and mixed with growth medium to produce 0.6% agar. The mixture was quickly plated and solidified for 45 min. Cells were suspended in 0.6 ml 2× growth medium and mixed gently with 0.6 ml 0.8% agar /medium. Overall 1 ml of cells with 0.4% agar/medium mixture was added onto plate for solidification. Colony formation was examined using stereo microscopy and analyzed (CellSens Dimension, Olympus, Shinjuku, Tokyo, Japan).

Flow cytometry analysis

Cells were collected and fixed with 70% ethanol. The cell pellet was then treated with 1% TritonX-100. Cells were subsequently resuspended in 50 μg/ml propidium iodide (Sigma, St Louis, MO, USA) containing RNaseI (Roche, Indianapolis, IN, USA) followed by analysis on the LSR II XW4400 workstation (BD Biosciences).

Immunofluorescence

After 48 h of transfection, cells were fixed with 4% paraformaldehyde and incubated with primary antibodies (1:250) overnight at 4 °C. After washing, cells were incubated with fluorescence-labeled secondary antibody (1:100). After washing, coverslips were placed on a glass slide using UltraCruz mounting medium (Santa Cruz) before fluorescence microscope examination.

Immunoprecipitation, ubiquitination and protein half-life assays

The cell lysate was obtained by using immunoprecipitation lysis buffer as described previously.[48] LSD1 or IgG antibodies were added to cell lysate. Protein G-plus agarose beads (Santa Cruz) or Flag-M2 affinity gel were collected and subjected to IB. HA-Ubiquitin, pcDNA3.1-Flag-HDAC5 or empty vector plasmids were co-transfected into cells for 38 h. Cells were then treated with 10 μM MG132 for 10 h and collected for immunoprecipitation assay with protein G-plus agarose beads. For half-life studies 48 h after transfection with pcDNA3.1-HDAC5 or HDAC5 siRNA, cells were treated with 100 μg/ml cycloheximide and then collected at indicated times for IB.

Protein acetylation assay

The immunoprecipitates of FLAG-M2 agarose from MDA-MB-231 cells overexpressing FLAG-tag USP28 or FLAG-tag LSD1 were used as substrates for the protein deacetylation assay. Pull-down of IgG was used as negative control. A total of 0.25 μg of recombinant human GST-tagged HDAC5 protein (Creative BioMart, NY, NY) was mixed with 30 μl immunoprecipitates or 1.5 μg bulk histone at 37 °C for 6 h in a buffer containing 40 mM Tris–HCl (pH 8.0), 2.5 mM $MgCl_2$, 50 mM NaCl, 2 mM KCl, 0.5 mM DTT, 1 mM EDTA and protease inhibitor. The reactions were then subjected to immunoblots with anti-acetyl lysine antibody (EMD Millipore, Billerica, MA, USA). FLAG-tagged USP28 or LSD1 and bulk histone were probed with anti-FLAG antibody or H3 antibody as loading control. Inactive HDAC5-GST protein was used as negative control by heating recombinant protein at 95 °C for 5 min. *In vivo* protein acetylation assay was performed using cell lysate of MDA-MB-231 cell transfected with scramble and HDAC5 siRNAs. LSD1 or IgG antibodies were added to cell lysate. Protein G-plus agarose beads (Santa Cruz) were collected and subjected to IB with anti-acetyl lysine or LSD1 antibodies.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assay was performed as described previously.[12] Primary antibodies against HDAC5, LSD1, H3K4me2 and acetyl-H3K9 were used as indicated for immunoprecipitation of the protein–DNA complexes. PCR primer sets used for amplification of precipitated fragments were shown in Supplementary Table S1. Input DNA was used for normalization.

Statistical analysis

Data were represented as the mean ± s.d of three independent experiments. The quantitative variables were analyzed by the two-tailed Student's *t*-test. The χ^2 study was used to assess the correlation between HDAC5 and LSD1 protein expression by using median H-scores as the cutoff for high- versus low-protein expression. *P*-value < 0.05 was considered statistically significant for all tests. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Functional characterization of lysine-specific demethylase 2 (LSD2/KDM1B) in breast cancer progression

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ABSTRACT

Flavin-dependent histone demethylases govern histone H3K4 methylation and act as important chromatin modulators that are extensively involved in regulation of DNA replication, gene transcription, DNA repair, and heterochromatin gene silencing. While the activities of lysine-specific demethylase 1 (LSD1/KDM1A) in facilitating breast cancer progression have been well characterized, the roles of its homolog LSD2 (KDM1B) in breast oncogenesis are relatively less understood. In this study, we showed that LSD2 protein level was significantly elevated in malignant breast cell lines compared with normal breast epithelial cell line. TCGA- Oncomine database showed that LSD2 expression is significantly higher in basal-like breast tumors compared to other breast cancer subtypes or normal breast tissue. Overexpression of LSD2 in MDA-MB-231 cells significantly altered the expression of key important epigenetic modifiers such as LSD1, HDAC1/2, and DNMT3B; promoted cellular proliferation; and augmented colony formation in soft agar; while attenuating motility and invasion. Conversely, siRNA-mediated depletion of endogenous LSD2 hindered growth of multiple breast cancer cell lines while shRNA-mediated LSD2 depletion augmented motility and invasion. Moreover, LSD2 overexpression in MDA-MB-231 cells facilitated mammosphere formation, enriched the subpopulation of $CD49f^{+}/EpCAM^{-}$ and $ALDH^{high}$, and induced the expression of pluripotent stem cell markers, NANOG and SOX2. In xenograft studies using immune-compromised mice, LSD2-overexpressing MDA-MB-231 cells displayed accelerated tumor growth but significantly fewer lung metastases than controls. Taken together, our findings provide novel insights into the critical and multifaceted roles of LSD2 in the regulation of breast cancer progression and cancer stem cell enrichment.

INTRODUCTION

Histone lysine methylation is an important covalent post-translational modification (PTM) of chromatin. Histone lysine methyltransferases (KMTs) and demethylases (KDMs) are groups of enzymes that have pivotal roles in dynamic regulation of numerous chromatin functions such as gene transcription, chromatin stability, DNA replication and repair [1, 2]. To date, two different classes of KDMs have been recognized: the flavin-dependent amine oxidase-containing and the Jumonji C (JmjC)-domain-containing enzymes. The flavin-dependent KDM family includes LSD1 (KDM1A) and LSD2 (KDM1B), which both contain a SWIRM domain and share significant sequence homology in their amine oxidase domains. However, LSD2 possesses an N-terminal zinc finger motif, which is required for binding to methylated histone lysine, while lacking LSD1's co-factor binding tower domain. Both enzymes oxidize Carbon-Nitrogen bonds with subsequent production of a demethylated substrate, lysine 4 of histone 3, in a flavin-dependent manner [3, 4]. Although LSD1 and LSD2 are highly similar in amino acid sequences, catalyzed chemical reactions, and substrates, it is evident that the two enzymes also have distinct functions, and therefore may act differentially in regulating chromatin structure and function. Moreover, while LSD1 is mainly associated with the promoter region of genes, LSD2 tends to bind at transcribed coding regions and does not assemble the same transcription repressor complexes as LSD1 [5, 6]. These findings suggest that LSD1 and LSD2 likely interact with different protein partners in the nucleus and play quite distinct roles in regulating key cellular processes.

In the past decade, the flavin-dependent demethylase family has emerged as a potential therapeutic target for breast cancer. According to the data from The Cancer Genome Atlas (TCGA) database, mRNA expression levels of both LSD1 and LSD2 are greatly increased in breast cancer patient specimens in comparison to normal breast tissues. A role for LSD1 has been consistently implicated in tumorigenesis in various cancers, including breast cancer [7-14]. Importantly, LSD1 expression is highly associated with a more aggressive breast cancer phenotype, and work from our laboratory and others has consistently shown LSD1 depletion hinders proliferation and metastasis of breast cancer cells [8, 11, 15, 16]. Many small molecule inhibitors targeting LSD1 have been developed in the past years, and antineoplastic efficacy of several promising compounds has been tested in clinical trials for treatment of cancers such as acute myeloid leukemia (AML) and lung cancer (http://clinicaltrials.gov).

LSD2 has been linked to numerous important biological processes including transcription regulation, chromatin remodeling, genomic imprinting, heterochromatin silencing, growth factor signaling and somatic cell reprogramming [6, 17-20]. While the roles of LSD2 in breast cancer biology have been emerging, the underlying mechanisms are still largely unknown. Recent studies from our laboratory demonstrated that inhibition of LSD2 attenuates colony formation and downregulates global DNA methylation in breast cancer cells [21]. Combined inhibition of DNA methyltransferase (DNMT) and LSD2 reactivates expression of abnormally silenced genes with important functions in breast cancer and enhances cellular apoptotic responses. These findings suggest that combinatorial therapy targeting LSD2 and DNMTs effectively improves the antitumor efficacy of DNMT inhibitors in breast cancer. In this report, we elucidate the *in vitro* and *in vivo* activities of LSD2 in regulation of breast cancer proliferation, migration, invasion and cancer stem cell propagation. These studies provide novel insight into the multifaceted roles of LSD2 in breast cancer progression.

RESULTS

LSD2 expression is elevated in breast cancer cell lines and clinical specimens

We examined LSD2 protein level in several human breast cancer cell lines and the normal immortalized human mammary epithelial cell line, MCF10A. Western blots showed that LSD2 protein expression is elevated in breast cancer cell lines compared with MCF10A cells (Figure 1A and 1B). Next, *in silico* analysis of LSD2 expression in clinical cancer patient samples indicated that compared with corresponding normal tissue counterparts, several cancer types including breast have significantly elevated LSD2 mRNA expression (Figure 1C, Supplementary Table 1) (TCGA PANCAN RSEM TPM data downloaded from https://toil.xenahubs.net). Overexpression of LSD2 in several pathological types of breast cancer was also found in METABRIC dataset (Curtis Breast) (Supplementary Table 2) (https://www.oncomine.org). Further analysis of LSD2 expression across all molecular subtypes of breast cancer showed that LSD2 mRNA level is significantly higher in basal-like tumors as compared to other breast cancer subtypes or normal tissues (Figure 1D) (TCGA data downloaded from GSE62944). Taken together, these data suggest a consistent increase of LSD2 expression in breast cancer cell lines and clinical tumor samples warranting further investigation into the role of LSD2 in breast cancer progression.

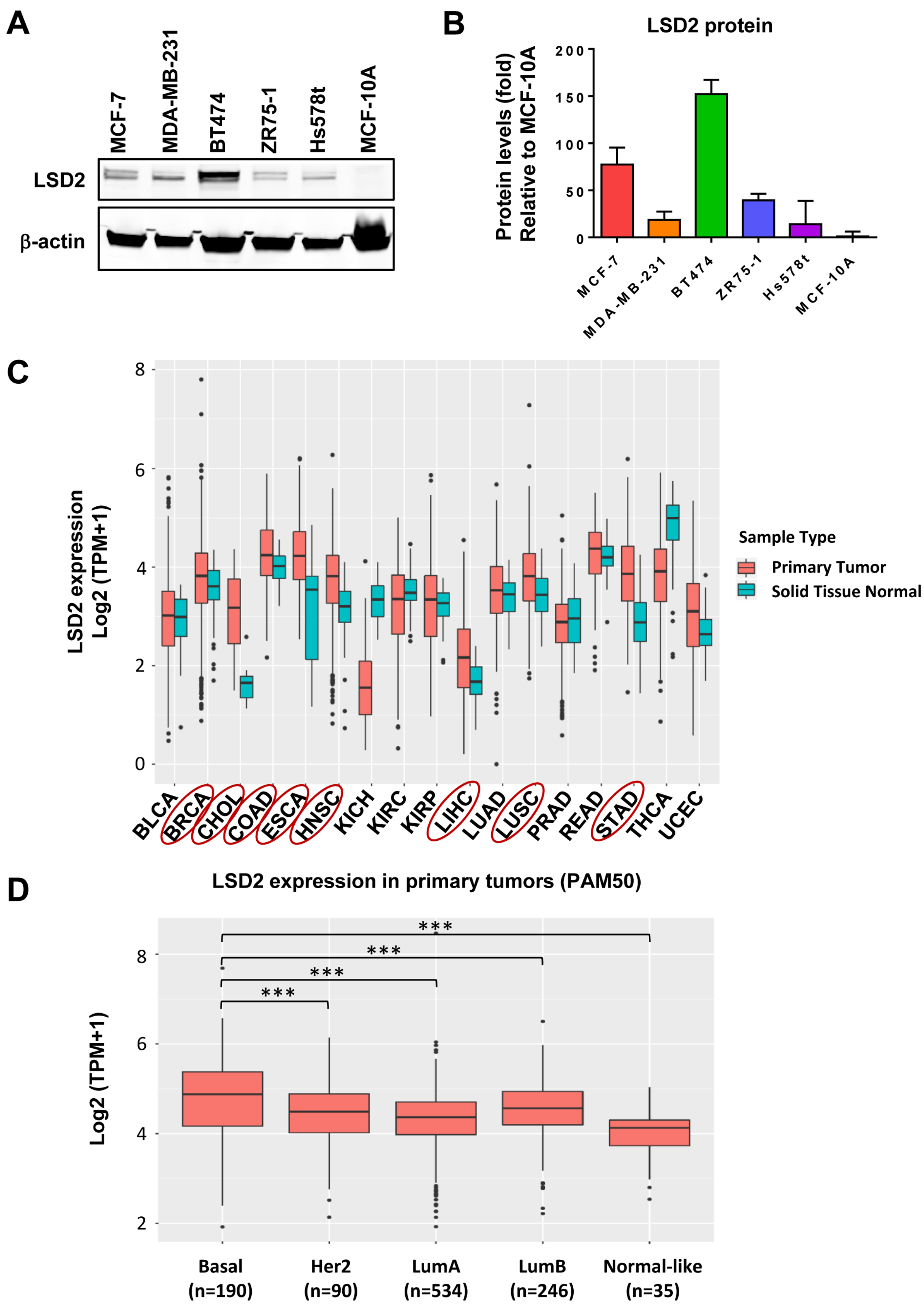

Figure 1: Expression level of LSD2 in breast cancer cell lines and clinical tumor specimens. A. Western blot examination of LSD2 protein expression in breast cancer and MCF10A cell lines. **B.** Quantification of western blot results of LSD2 expression. **C.** TCGA data analysis of mRNA level of LSD2 in different types of cancer. Cancer types with significantly elevated LSD2 mRNA level were highlighted with Red circle. P-values were calculated using Mann-Whitney U test and corrected for multiple comparisons using Benjamini-Hochberg. **D.** mRNA levels of LSD2 in different subtypes of breast cancer. Tukey multiple comparisons of means, *** $p < 0.001$.

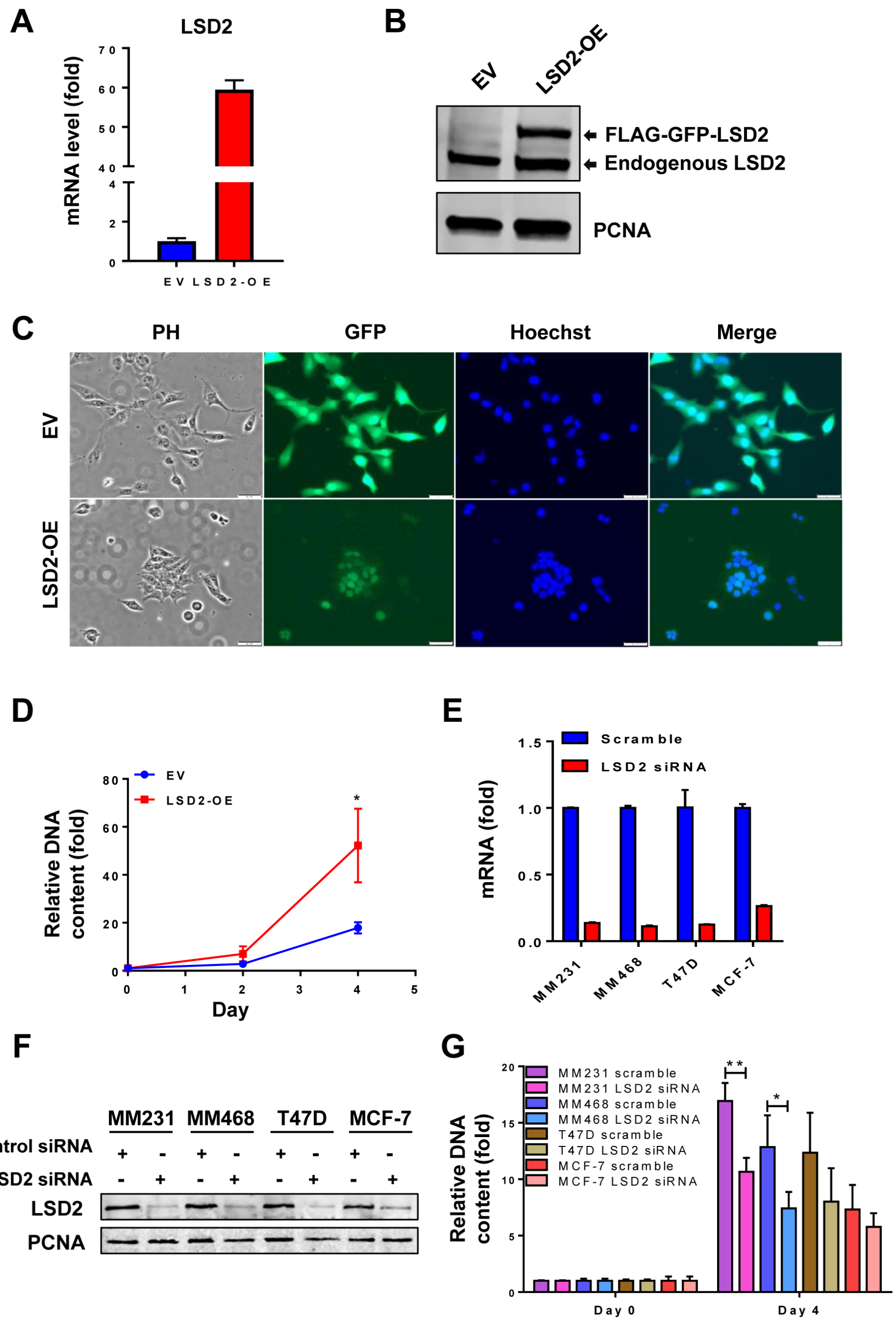

Figure 2: Effect of LSD2 overexpression or depletion on proliferation of breast cancer cells. A. MDA-MB-231 cells were transfected with control empty vector (EV) or LSD2 overexpression vector (OE) for 48 h followed by selection with G418. mRNA expression of LSD2 was measured by quantitative real-time PCR with GAPDH as an internal control. **B.** Cellular nuclear proteins were extracted, and LSD2 protein expression in MDA-MB-231-EV or LSD2-OE cells was examined by Western blots using anti-LSD2 antibody with proliferating cell nuclear antigen (PCNA) as an internal control. **C.** MDA-MB-231 cells transfected with control empty vector (EV) or LSD2 overexpression vector (LSD2-OE) were fixed with 4% PFA followed by Hoechst 33258 staining. Bright field and fluorescent images were taken to observe cellular morphology and LSD2-GFP protein expression. PH, Phase Contrast. **D.** MDA-MB-231 cells transfected with control empty vector (EV) or LSD2 overexpression vector (LSD2-OE) were analyzed for growth using fluorometric dsDNA quantitation method. **E.** Human breast cancer MDA-MB-231, MDA-MB-468, MCF-7 and T47D cells were transfected with scramble or LSD2 siRNA for 96 h followed by qPCR examination of LSD2 mRNA expression level. β-actin was used as an internal control. **F.** Cells transfected with scramble or LSD2 siRNA were examined for LSD2 protein expression by western blots with PCNA as an internal control. **G.** Fluorometric dsDNA quantitation assays were performed to evaluate growth of breast cancer cells which were transfected with scramble or LSD2 siRNA for 96 h. All experiments were performed at least three times and bars represent the means of three independent experiments ± s.d. * $p < 0.05$, ** $p < 0.01$, Student's t-test.

LSD2 promotes breast cancer cell growth and colony formation

To explore the functional role of LSD2 in regulating breast cancer development, we stably overexpressed eGFP and Flag-dually tagged LSD2 in MDA-MB-231 (LSD2-OE) and validated the overexpression at the mRNA and protein levels (Figure 2A and 2B). Tracking of the GFP tag through fluorescent microscopy showed that the LSD2-eGFP-Flag localizes exclusively to the nucleus in MDA-MB-231 cells (Figure 2C). While cells transfected with control empty vector (EV) display the spindle shaped morphology of parental MDA-MB-231 cells, LSD2 overexpression induces a cobblestone-like morphology with apparent cell-cell adhesion (Figure 2C).

Next, we investigated the potential impact of increased LSD2 expression on breast cancer cell proliferation. Cellular proliferation assays showed that stable overexpression of LSD2 in MDA-MB-231 cells significantly promoted cellular growth rate (Figure 2D). To further validate this phenotypic change, two basal-like/triple-negative breast cancer (TNBC) cell lines, MDA-MB-231 and MDA-MB-468, and two luminal/Estrogen Receptor positive (ER+) cell lines, T47D and MCF-7, were transfected with non-targeting scramble or LSD2-specific siRNA. LSD2-targeting siRNA effectively suppressed endogenous LSD2 mRNA and protein expression in all lines (Figure 2E and 2F). Although depletion of LSD2 hindered the cell proliferation in all lines, this effect was more pronounced and statistically significant in TNBC cell lines as compared to ER+ cell lines (Figure 2G).

Our previous study demonstrated that shRNA-mediated inhibition of LSD2 leads to a significant reduction in 2D colony formation in MDA-MB-231 cells, indicating a survival-promoting role for LSD2 in breast cancer cells [21]. In this study, we investigated the effect of LSD2 overexpression on 2D colony formation of MDA-MB-231 cells. In agreement with the effect of LSD2 knockdown, ectopic expression of LSD2 in MDA-

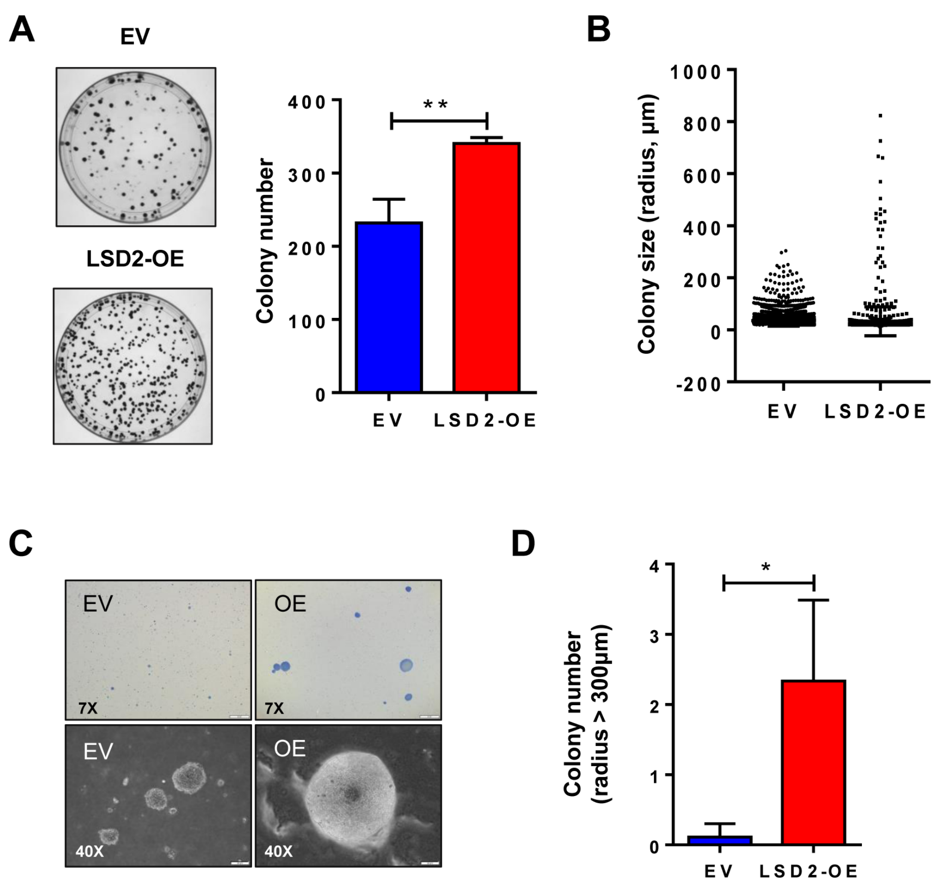

Figure 3: LSD2 enhances the colony formation capacity of MDA-MB-231 cells. A. 500 cells stably transfected with empty vector or LSD2 expression plasmids were plated in 10cm dish. After 14 days, colonies formed were stained with 0.5% crystal violet and counted. **B.** 10,000 cells per dish were seeded in 0.4% soft agar in 35mm dish. After 3 weeks, colonies were stained with 0.005% crystal violet and counted using CellSens software. Individual colonies formed by empty vector control or LSD2 overexpressing MDA-MB-231 cells were plotted based to colony size (μm). **C.** Representative microscopy images (7x and 40x) of cellular colonies after 3 weeks of seeding the cells on soft agar coated wells. **D.** Average numbers of colony whose radius is over 300 mm. Error bar represents ± s.d. from three independent experiments. * $p < 0.05$, ** $p < 0.01$, Student's t-test.

MB-231 cells significantly increases the number of 2D colonies (Figure 3A). We then extended our investigation to an anchorage-independent soft-agar colony formation assay to further dissect the role of LSD2 in breast tumorigenicity. The soft agar results showed that, although there was no significant difference in average colony size (Figure 3B), LSD2-OE cells developed an increased number of larger colonies (> 300 µm) than empty vector cells (Figures 3B, 3C and 3D). Collectively, these results suggest that LSD2 enhances *in vitro* colony formation capacity of breast tumor cells.

LSD2 attenuates motility and invasion of breast cancer cells

Enhanced motility and invasion are positively associated with the aggressive behavior and poor prognosis of breast cancer. We anticipated that accelerated growth rate by LSD2 overexpression would lead to corresponding augmentation of cellular motility and invasion and tested this hypothesis through transwell Boyden chamber assays. Unexpectedly, we found that LSD2 overexpression significantly reduced migration and invasion of MDA-MB-231 cells (Figure 4A and 4B). To validate this result, we performed the same experiments using a pool of MDA-MB-231 cells stably expressing shRNA against LSD2, which decreased LSD2 mRNA expression by about 75% as compared with scramble control cells (Supplementary Figure 1). Boyden chamber assays demonstrated that loss of LSD2 facilitated cell migration and invasion of MDA-MB-231 cells (Figure 4C and 4D). To further verify these results, we performed *in vitro* wound-healing assay and found that MDA-MB-231 cells transfected with control empty vector closed the wound much more efficiently than LSD2-overexpressing cells (Figure 4E and 4F). On the contrary, inhibition of LSD2 in MDA-MB-231 cells significantly augmented the wound-healing rate (Figure 4G and 4H). Collectively, these results point to an inhibitory role of LSD2 in mediating breast cancer cell migration and invasion.

LSD2 overexpression promotes breast cancer stem cell-like characteristics

Breast cancer stem-like cells (BCSCs) possess features of multipotent, oncogenic, and self-renewal capacity, which are responsible for breast tumor heterogeneity [22, 23]. Recent studies have shown that LSD1 plays a critical role in promoting the differentiation and self-renewal of cancer stem cells (CSCs) in human breast cancer and in other cancer types [24, 25]. To elucidate the potential implication of LSD2 in breast cancer stem cell phenotypes, mammosphere formation assay was carried out, which showed that LSD2 overexpression significantly increases the size and number of both primary and tertiary spheres (Figure 5A and 5B), suggesting the enrichment of a subpopulation of CSCs with self-renewal capacity in LSD2-OE cells. Flow cytometry analysis of LSD2-OE cells indicated a significantly increased CD49f+/EpCAM- subpopulation, which is considered to be enriched for stem/basal progenitor cells (Figure 5C and 5D). We also examined the nuclear protein expression of four embryonic stem cell (ESC) markers, KLF4, NANOG, OCT4 and SOX2 and observed that LSD2 overexpression increases expression of NANOG and SOX2 (Figure 5E and 5F). Finally, we investigated the level and activity of Aldehyde Dehydrogenase (ALDH) in LSD2-OE cells. Recent studies indicate that enhanced ALDH activity is a hallmark of cancer stem cells [26, 27]. In line with previous report that MDA-MB-231 cells express very low level of ALDH (0%-1% positive) [28], no obvious ALDH^{high} cells were detected in MDA-MB-231 EV cells (around 0%) whereas LSD2 overexpression increased ALDH^{high} cell population to about 1.5% (Supplementary Figure 2). In addition, mRNA expression of many ALDH family members was increased by LSD2-OE based on our recently microarray study (Supplementary Table 3). Collectively, all these data point to the critical function of LSD2 in promoting BCSC-like properties.

Overexpression of LSD2 alters expression of key epigenetic modifiers

Our recent studies have revealed that dysregulated regulatory networks formed by aberrant crosstalk between histone methylation and histone acetylation or DNA methylation profoundly impact breast cancer progression [13, 15, 21, 29]. To explore the involvement of LSD2 in these regulatory processes, we assessed the impact of LSD2 overexpression or deficiency on mRNA and protein expression of key members of DNMT, HDAC and KDM families. Quantitative RT-PCR results showed that LSD2 overexpression significantly increased the mRNA levels of LSD1, HDAC1, 2, 3, 5, 6, 8, DNMT3B and 3L, KDM4B and KDM5B (Figure 6A). On the other hand, expression of only a few genes was affected by LSD2 stable knockdown, including HDAC9 and DNMT3L (Figure 6B). In LSD2 siRNA-transfected MDA-MB-231 cells, mRNA levels of LSD1, HDAC4, and DNMT3B were decreased while HDAC1 mRNA level was increased (Supplementary Figure 3). The protein expression of several genes was further tested to determine if there is correlated alteration between mRNA and protein expression. Quantitative western blots showed that LSD2 overexpression significantly increased the protein expression of LSD1, HDAC1, 2, 6, 8 and DNMT3B, and inhibited the expression of HDAC5 and DNMT3L (Figure 6C), whereas DNMT3B was the only factor altered by LSD2-KD (Figure 6D).

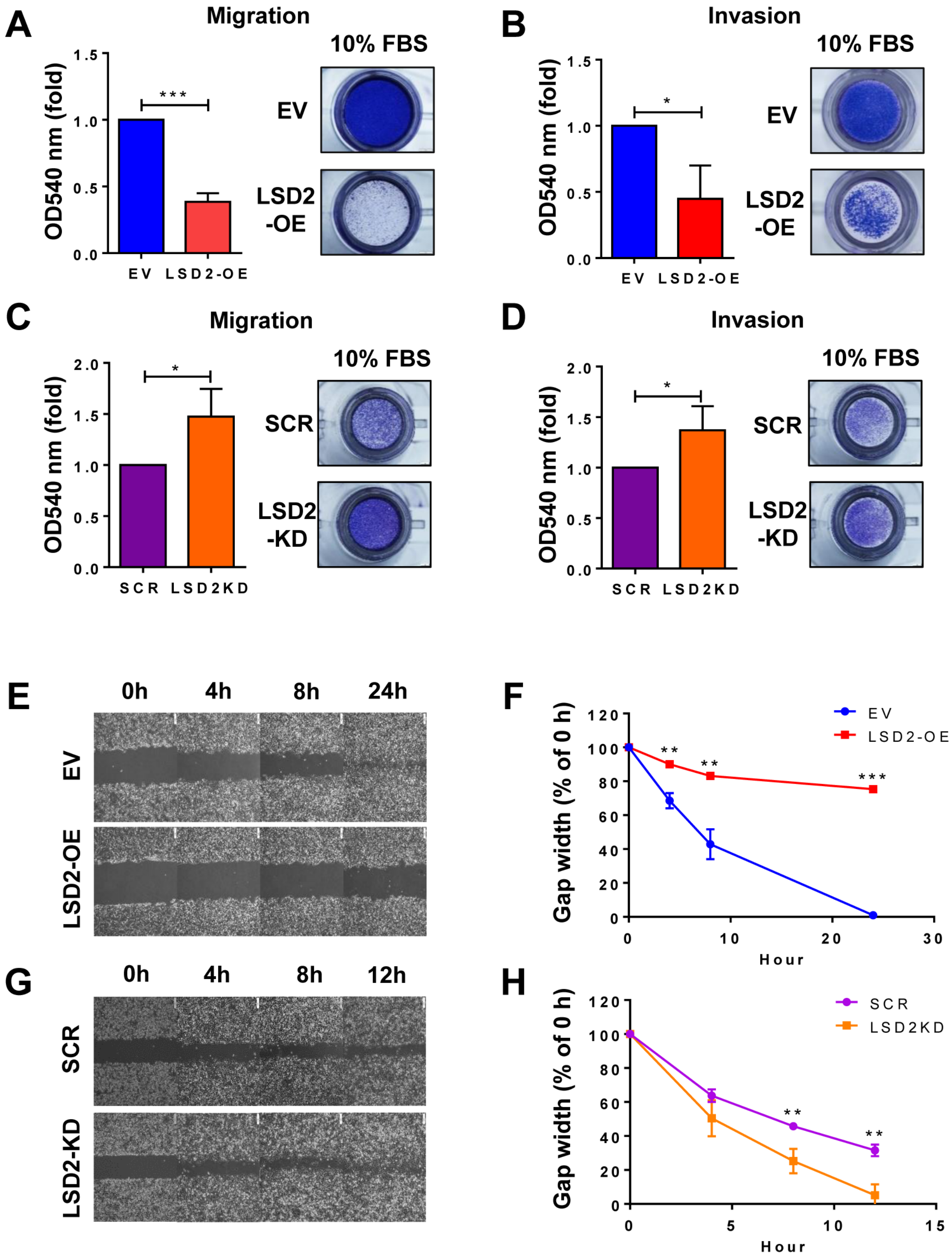

Figure 4: LSD2 regulates migration and invasion in MDA-MB-231 cells. A. Transwell migration assay was performed to detect the migratory capacity of MDA-MB-231 EV and LSD2-OE cells. Quantification of the migrated cells was done by solubilization of crystal violet and spectrophotometric reading at OD 540. **B.** Quantification of the invasive MDA-MB-231 EV and LSD2-OE cells. Transwell invasion assay was performed and the invasive cells were quantified by solubilization of crystal violet and spectrophotometric reading at OD 540. **C.** Quantification of the migratory MDA-MB-231 cells transfected with scramble and LSD2 shRNA plasmids. **D.** Quantification of the invasive MDA-MB-231 cells transfected with scramble and LSD2 shRNA plasmids. **E.** Confluent monolayers of EV and LSD2-OE MDA-MB-231 cells were wounded by scratch with a pipette tip. Cells were then incubated for 24 h. Images were taken at the end points to be compared to 0 h to measure wound healing. **F.** The average of wound closure rate during the first 24 h of wound healing was calculated. **G.** Confluent monolayers of scramble shRNA and LSD2-KD MDA-MB-231 cells were wounded by scratch. Cells were then incubated for 12h. Images were taken at the end points to be compared to 0 h to measure wound healing. **H.** The average of wound closure rate during the first 12 h of wound healing was measured and quantified. All experiments were independently performed at least three times and values represent the mean ± s.d. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t-test.

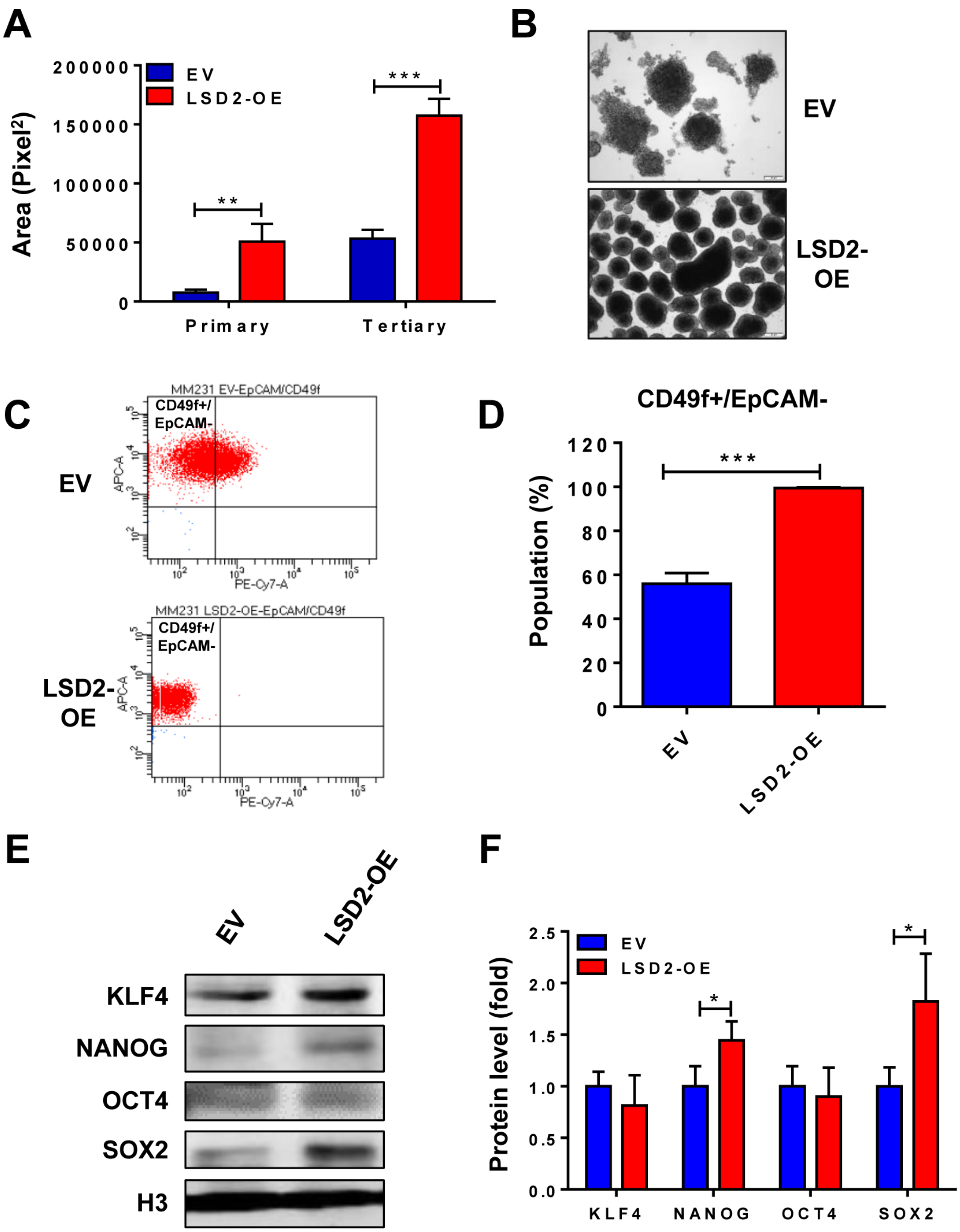

Figure 5: Overexpression of LSD2 facilitates breast cancer stem cell characteristics. A. MDA-MB-231 EV or LSD2-OE cells were suspended in tumor sphere medium and seeded in 6-well plate with ultra-low attachment surface. After 7-day incubation, spheres were collected and digested into single cells. Same density of digested cells was seeded for secondary mammosphere and tertiary mammosphere formation. Quantification of primary and tertiary mammospheres was performed using CellSens software. **B.** Representative pictures of tertiary mammospheres formed by EV and LSD2-OE cells. **C.** Flow cytometry analysis of cell surface marker CD49f and EpCAM in EV and LSD2-OE cells. **D.** The percentage of $CD49f^{+}/EpCAM^{-}$ cells was quantified from three independent experiments. **E.** Western blot examinations on nuclear protein levels of KLF4, NANOG, OCT4 and SOX2 in EV and LSD2-OE cells. Histone 3 (H3) was used as internal control. **F**. The experiments were performed three times with similar results. Values represent means ± s.d. * $p < 0.05$, *** $p < 0.001$, Student's t-test.

Increased LSD1 expression in LSD2-OE cells raises an important question as to whether the tumor growth promoting activities of LSD1 and LSD2 are interdependent. To address this question, a rescue experiment was carried out to knock down LSD1 expression by siRNA in EV and LSD2-OE cells. Treatment with siRNA effectively depleted the mRNA expression of LSD1 without altering LSD2 expression levels (Supplementary Figure 4). Rescue with LSD1 siRNA hindered the growth of both MDA-MB-231 EV and LSD2-OE cells, but exhibited a similar extent of rescue efficiency (decreases of about 35% vs 39%) (Figure 6E). This result clearly indicates that LSD2 promotes breast cancer cell proliferation in an LSD1-independent manner.

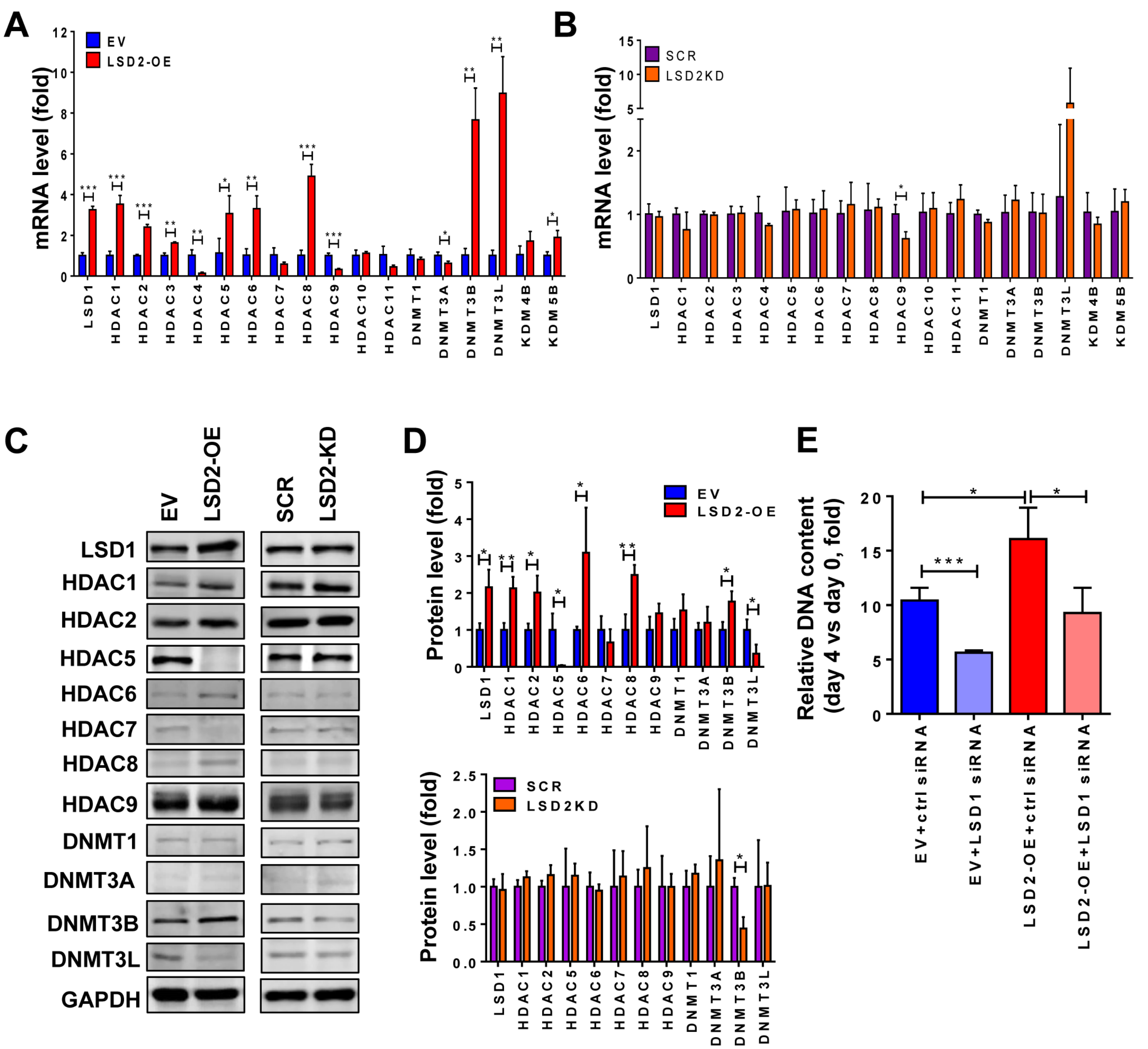

Figure 6: Effect of LSD2 on expression of key epigenetic modifiers. **A.** RNA was extracted from MDA-MB-231 EV and LSD2-OE cells and cDNA was synthesized and subjected to quantitative real-time PCR for the indicated genes using TaqMan probes. GAPDH expression was used as an internal standard. **B.** mRNA expression of chromatin modifying factors in MDA-MB-231 cells stably transfected with scramble (SCR) or LSD2 shRNA (LSD2-KD). GAPDH expression was used as an internal standard. **C.** Indicated chromatin modifying factors were analyzed for their protein levels by western blots in MDA-MB-231 EV, LSD2-OE, scramble shRNA and LSD2-KD cells. GAPDH was used as a loading control. **D.** Histograms represent the average protein levels of indicated chromatin modifiers in three independent experiments relative to GAPDH protein ± s.d. as determined by quantitative immunoblots. **E.** MDA-MB-231 EV and LSD2-OE cells were transfected with scramble or LSD1 targeting siRNA for 96 h followed by growth assay using fluorometric dsDNA quantitation. Column with error bar represents mean ± s.d. from three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p < 0.001$, Student's t-test.

Overexpression of LSD2 promotes growth and inhibits lung metastasis of MDA-MB-231 xenograft tumors in nude mice

To confirm our *in vitro* results, we implanted MDA-MB-231 EV and LSD2-OE cells into the mammary fat pads of athymic nude mice. LSD2 overexpression led to accelerated tumor growth, with approximately three-fold increase in average tumor size over empty vector cells (Figure 7A and 7B). Statistical analysis of *in vivo* tumor growth is summarized in Supplementary Table 4. Average weight of LSD2-OE tumors was statistically higher than control group at the end of the experiment (Figure 7C). Both groups of animals had normal body weight gains (Figure 7D). To evaluate *in vivo* effect of LSD2 on tumor metastasis, we quantified mRNA expression of human housekeeping gene HPRT1 in mouse lung tissue samples by real-time RT-PCR using a probe that does not cross-react with its mouse counterpart. Our results showed that

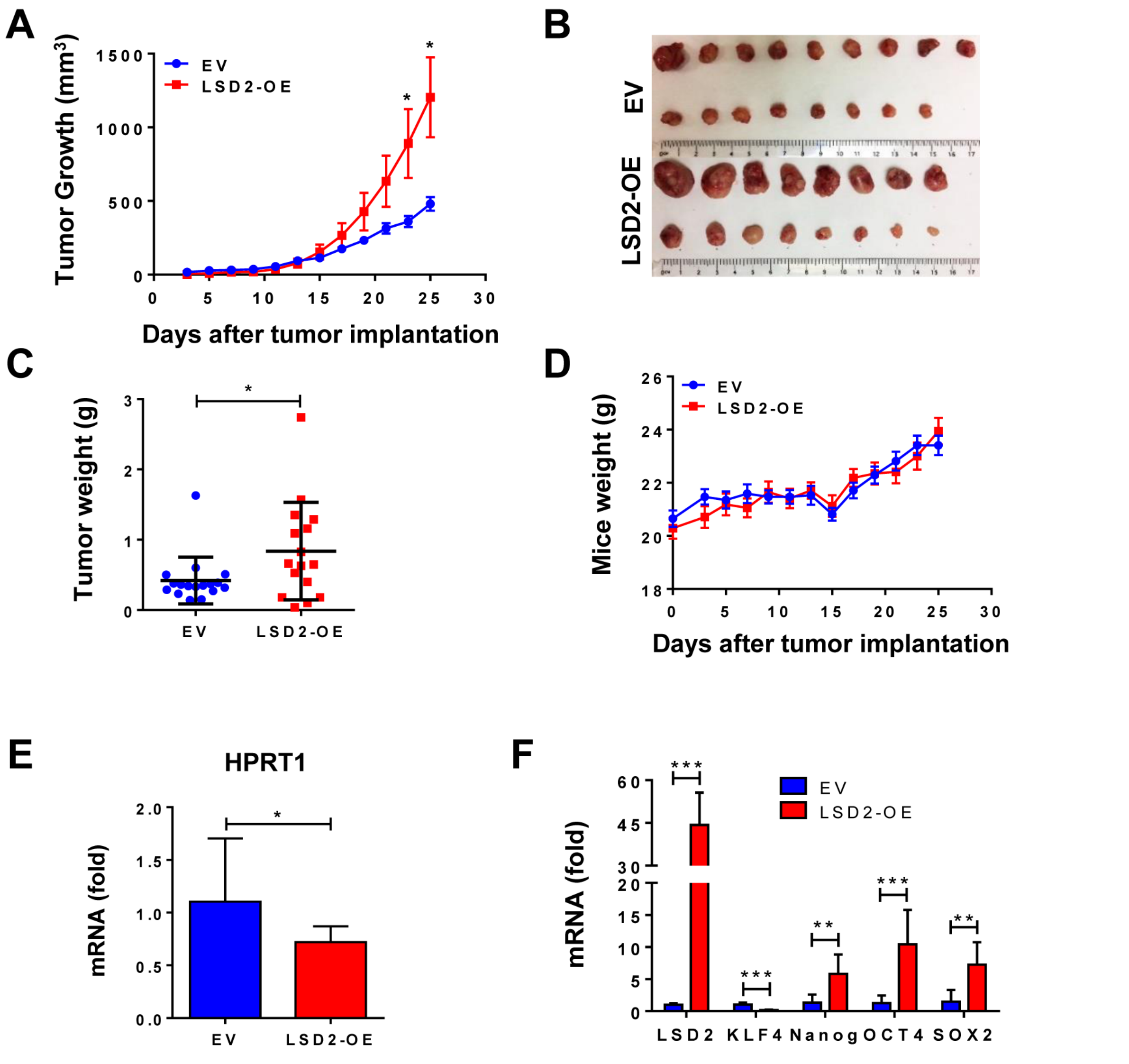

Figure 7: *In vivo* effect of LSD2 on proliferation and metastasis in mice bearing MDA-MB-231 xenograft. A. MDA-MB-231 cells transfected with empty vectors ($n = 17$) or LSD2 expression vectors ($n = 16$) were transplanted into the mammary fat pad of nude mice. Tumor volumes were regularly assessed every two days. Shown are average tumor volumes ± s.e. **B.** Orthotopically implanted tumors were removed after terminating the experiments. Shown are pictures of implanted tumors. **C.** Weight of individual animal tumor was measured at the end of experiment. **D.** Weights of mice were measured on the indicated days. Points, mean mouse weight (g); bars, mean ± s.d. **E.** Tumor cells metastasized to mice lung were assessed by quantification of mRNA expression of human HPRT1 gene (EV, $n = 10$; LSD2-OE, $n = 16$). Mouse b-actin was used as internal control. Graph was plotted as fold change with normalization to EV. **F.** Total RNA was extracted from 7 randomly selected tumors from each group and mRNA levels of the four embryonic stem cell markers were evaluated by qPCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's t-test.

mRNA level of hHPRT1 gene was significantly reduced in lung tissues of mice bearing LSD2-OE tumors (Figure 7E). Normal mouse lung tissue was used as a negative control, and no expression of hHPRT1 was detected, thus validating the specificity of the hHPRT1 probe (Data not shown). To determine the *in vivo* impact of LSD2 overexpression on cancer stem cell markers, qPCR analysis was performed on RNA from tumors, which showed that the mRNA expression of NANOG, OCT4 and SOX2 were significantly induced in LSD2-OE xenograft tumor cells (Figure 7F). In agreement with *in vitro* results, the findings from this mouse study suggest that LSD2 promotes breast tumor growth and BCSC characteristics, while simultaneously attenuating cell invasion and dissemination *in vivo*.

DISCUSSION

Histone demethylases have emerged as a novel class of epigenetic regulators controlling cancer initiation and progression [30]. Dysregulated expression and functions of histone lysine demethylases are found in many types of cancers, and thus represent novel promising therapeutic targets for cancer. In the past decade, rapid progress has been made in understanding the molecular basis of histone demethylase-dependent functions in breast cancer biology [16, 20]. Among these enzymes, LSD1 is the first recognized histone lysine demethylase and perhaps one of the best-characterized histone-targeted enzymes in breast cancer. However, the involvement of LSD2, the only identified homolog of LSD1, in breast cancer is still very elusive. *In silico* data indicate a significant elevation of LSD2 expression in aggressive basal-like breast tumors as compared with other breast cancer subtypes and normal tissues, suggesting a potential link between LSD2 overexpression and aggressiveness of breast cancer. However, the molecular mechanism of LSD2 upregulation in breast cancer and the long-term clinical impact of elevated LSD2 expression in the risk stratification of breast cancer patients are still unclear. Therefore, more robust studies are needed to clarify these questions.

While LSD1 is typically associated with oncogenic phenotypes in almost all types of cancer, little is known about the function of LSD2 in mediating tumor progression. A recent study by Yang *et al* reported that LSD2 acts as an E3 ubiquitin ligase and inhibits A549 lung cancer cell growth through proteasomal degradation of O-GlcNAc transferase (OGT) [31], suggesting that LSD2 may inhibit the growth of certain types of cancer in a ubiquitination-dependent manner. The *in vivo* effect of LSD2 on A549 cell growth warrants further examination. In our study, we utilized both *in vitro* and *in vivo* models to investigate the potential implication of LSD2 in regulating breast cancer proliferation and metastasis. We found that overexpression of LSD2 in breast cancer cells consistently enhances MDA-MB-231 cell growth *in vitro* as well as in tumor xenografts in mice, whereas depletion of LSD2 by siRNA hinders the growth of multiple breast cancer cell lines. We also showed that LSD2 overexpression increases the number of colonies in 2D monolayer culture and large colonies in anchorage-independent 3D culture, indicating that LSD2 may potentiate the malignant transformative capacity of breast cancer cells. Interestingly, overexpression of LSD2 results in an increase of mRNA and protein expression of LSD1. A rescue study demonstrated that simultaneous treatment with LSD1 siRNA in control and LSD2-OE cells exerts similar effect on LSD2-mediated tumor cell growth. This result suggests that LSD1 and LSD2 may have non-redundant roles in promoting breast cancer proliferation.

The concept of breast cancer stem cells (BCSCs) was first introduced by Al-Hajj *et al* [32]. BCSCs are a rare subpopulation that originates from a small fraction of tumor initiating cells with the abilities of self-renewal, unlimited propagation and multipotent differentiation. Importantly, BCSCs are associated with poorer clinical outcome and are intrinsically resistant to therapy. Wu *et al* recently reported that the deubiquitinase USP28 promotes breast cancer stem cell (BCSC)-like characteristics *in vitro* and *in vivo* through stabilizing LSD1 protein [24]. We explored the potential regulation of LSD2 on BCSC features and showed that LSD2 overexpression facilitates the formation of several generations of mammospheres, enriches the $CD49f^{+}/EpCAM^{-}$ stem/basal progenitor subpopulation and promotes the expression of several pluripotent stem cell markers *in vitro* and in MDA-MB-231 xenograft tumors. Our findings indicate that, like LSD1, LSD2 has an important role in conferring CSC-like traits to breast cancer cells. In ESCs, the histone modification landscape profoundly influences the crosstalk of transcriptional regulators [33, 34]. Increasing lines of evidence suggest that the two key histone marks, H3K4 methylation and H3K27 methylation, serve as critical histone bivalent marks controlling developmental regulatory genes in embryos and ESCs [33, 35, 36]. LSD1 has been shown to act as a key histone modifier in the maintenance of pluripotency by occupying the promoter of a subset of developmental genes containing bivalent domains (H3K4 di/trimethylation and H3K27 trimethylation marks) and regulating the balance between self-renewal and differentiation in human ESCs [37]. It is probable that LSD2, in collaboration with LSD1, provides an additional layer of epigenetic modification in governing breast cancer stem cell features through modulation of the level of H3K4 methylation at pluripotent regulatory genes. Future study using genome-wide mapping approaches would aid in probing the subset of LSD2 target genes and histone mark alterations that are associated with biological processes in BCSC development.

Our studies point to potentially opposite roles of LSD2 in regulating breast cancer cell growth and invasion. Our *in vivo* study validated *in vitro* results showing that

lung metastasis is attenuated in mice bearing LSD2-overexpressing tumors. This opposite effect may reflect a broad and complex involvement of LSD2 in regulating histone function and gene transcriptional activities that could ultimately up-regulate growth-associated gene expression, while suppressing motility and invasion genes. Indeed, several other studies have reported that a number of genes possess opposite effects on cancer proliferation and metastasis [38, 39]. Morphologically, MDA-MB-231 LSD2-OE cells acquire tightly cohesive, cobblestone-like epithelial cell morphology as compared to the elongated fibroblast-like control cells. This finding suggests that increased LSD2 expression may induce a mesenchymal-epithelial transition (MET) through acquisition of epithelial markers with concurrent loss of mesenchymal features, which in turn leads to loss of migratory and invasive ability of tumor cells. Indeed, a number of genes involved in tight junction or apical-basal polarity such as OCLN, DSP, SCRIB, etc., were upregulated by LSD2-OE while VIM and FN1 were downregulated according to results of our recent microarray analysis (Supplementary Table 5). Some early studies have revealed that activated EMT program in non-transformed epithelial cells could confer properties of stem cells which may facilitate the development of tumor initiating cells [40]. However, a number of groups have recently reported that EMT may not be necessarily associated with cancer stemness features. For example, Schmidt *et al*., have shown that activities of EMT and stemness are somehow antagonistic and attenuation of the EMT process is required for the full acquisition of stem cell properties [41]. The Weinberg lab demonstrated that the EMT program may not be sufficient to induce changes of stemness in differentiated luminal cells, and additional genetic programs are needed to interact with EMT environment to induce phenotypic alteration of cancer stemness [42]. Future studies using appropriate *in vitro* and *in vivo* models are required to completely understand the precise role of LSD2 in regulating cross-talk between EMT/MET and stemness and its relevance in breast cancer progression and metastasis.

Our study also revealed that the expression levels of many key chromatin modifiers are altered by LSD2 overexpression, indicating a significant role of LSD2 in the epigenetic regulatory network in breast cancer cells. For example, stable LSD2 overexpression significantly increases the expression of LSD1, HDAC1, and HDAC2, which are important components of the NuRD (nucleosome remodeling and histone deacetylase) complex that has important implications in cancer biology [43, 44]. LSD2 overexpression also promotes the expression of DNMT3B, which is a critical epigenetic player in inducing aberrant DNA methylation and gene silencing in cancer [45]. The molecular mechanisms linking LSD2 to transcriptional regulation remain elusive. A study by Fang *et al* used ChIP-chip tiling array to map LSD2 binding loci on a genome-wide scale and found that, in addition to H3K4 demethylase activity, LSD2 may act as a positive regulator of gene transcription through binding to highly transcribed coding regions enriched in active histone marks such as H3K36me3 [6]. They also reported that LSD2 forms a complex with euchromatic histone methyltransferases EHMT1/2 and NSD3 as well as active transcription elongation factors such as Pol II and cyclin T1 [6]. We also noted that stable and transient knockdown of LSD2 exerted distinct impact on expression of epigenetic modifiers. It is possible that long-term suppression of LSD2 may intrinsically alter the genomic expression of other proteins and leads cells to compensate by increasing or reducing the expression of other signaling proteins. Further investigation is required to define the exact mechanisms by which LSD2 alters transcription of key epigenetic modifiers through mediating histone disassembly/reassembly and transcription elongation at gene coding regions.

In summary, our studies provide novel insight into the previously unrecognized roles of LSD2 in human breast cancer cells. We have shown for the first time that LSD2 augments proliferative and cancer stem cell traits, and attenuates motility and invasiveness of breast cancer cells. All of these findings suggest that LSD2 has complex and multifaceted roles in breast oncogenesis. In the future, better understanding of epigenetic downstream target genes and pathways controlled by LSD2 would aid in developing novel small molecule inhibitors and combination strategies which might confer selective effects against breast cancer.

MATERIALS AND METHODS

Cell lines and culture conditions

Human breast cancer cell lines MDA-MB-231, MDA-MB-468, MCF-7, T47D and normal immortalized breast epithelial cell line, MCF10A, were obtained from the ATCC/NCI Breast Cancer SPORE program. Cells were cultured in growth medium as described previously [15, 46]. Stable transfectant lines were maintained with 800 µg/mL G418 (Geneticin).

Plasmid construction and stable transfection

Full length human LSD2 cDNA from MCF-7 cells was originally cloned by PCR into pcDNA3.1/V5-His TOPO. PCR primers engineered with KpnI sites were used to amplify LSD2 and then cloned into eGFP-Flag vector (using KpnI site in MC1) purchased from Gene Copoeia (Rockville, MD). Empty eGFP-flag vector (EV) or LSD2-eGFP-Flag (LSD2-OE) was transfected into MDA-MB-231 cells using Lipofectamine 3000 (Thermo Fisher

Scientific, Waltham, MA) according to manufacturer's instructions. After 48-hour transfection, cells were selected with 800 μg/mL G418 for several weeks. Then eGFP-positive cells were further sorted three times by flow cytometry to enrich LSD2-eGFP-Flag overexpressing cells.

Small interfering RNA treatment

Pre-designed LSD2 or LSD1 siRNA and non-targeting scramble siRNA (Santa Cruz Biotechnology, Dallas, TX) were transfected into cells following manufacturer's protocol. Briefly, cells were seeded in 96-well plates the day before transfection. siRNA was prepared in transfection medium (sc-36868) with transfection reagent (sc-29528). Cells were washed using transfection medium before 100 μL of siRNA complexes were added. After 5-hour incubation at 37°C, 100 μL normal growth medium containing 2x fetal bovine serum was added to each well. After 96-hour incubation, relative cell number was evaluated using FluoReporter Blue Fluorometric dsDNA Quantitation Kit (Thermo Fisher Scientific) according to manufacturer's protocol.

shRNA treatment and stable cell line generation

Scramble and 4 different LSD2 shRNAs were purchased from SABiosciences (Germantown, MD) and reverse transfected with Attractene transfection reagent (using GFP expression plasmids first, followed by Gentamycin expression plasmids) into MDA-MB-231 cells. At 48 h post-transfection, cells were first selected with 800 μg/ml G418 for several weeks, and then sorted by flow cytometry to enrich for GFP+ cells. All transfections were assayed by qPCR and western blot analysis for the best knockdown efficiency.

RNA extraction and qPCR

Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) following manufacturer's instructions. Tissues were directly homogenized in RNA lysis buffer which in this kit is RLT buffer. cDNA was synthesized using M-MLV Reverse Transcriptase (Thermo Fisher Scientific). Quantitative real-time PCR was performed on the StepOne real-time PCR system using TaqMan Gene Expression Assays (ThermoFisher Scientific).

Immunoblotting

Whole cell lysate and nuclear proteins were extracted as described previously [15, 21, 29]. Briefly, 60 μg whole cellular protein or 30 μg nuclear protein was separated on Mini-PROTEAN® TGX™ 4-20% acrylamide gels and transferred onto NC membranes. Antibodies used in this study are shown in Supplementary Table 6. CD49f-APC and EpCAM-PE-Cy7 antibodies (BD Biosciences, Franklin Lakes, NJ) were provided by Dr. Mei Zhang (University of Pittsburgh Cancer Institute). Membranes were scanned with Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Cell proliferation assay

Cells were seeded at 1000 to 5000 cells per well in 96-well plates. At each time point, medium was discarded by inverting the plates. Then the plates were frozen in -80°C freezer until ready to be measured. 100 μl distilled water was added into each well after the plates were thawed to room temperature. Then the plates with water were incubated at 37°C for 1h. Plates were frozen and thawed again to lyse the cells in order to release DNA completely. The DNA content was measured using FluoReporter Blue Fluorometric dsDNA Quantitation Kit (Thermo Fisher Scientific) by adding 100 μL of aqueous Hoechst 33258 in TNE buffer into each well and then measured using VICTOR X4 plate reader (PerkinElmer, Waltham, MA).

Monolayer culture colony formation assay

Empty vector and LSD2-OE MM231 cells were seeded at 500 cells per 10cm dish. After 14 days, cells were stained with 0.5% crystal violet, dried overnight and colonies were counted. Colonies that contained >50 cells were scored. All experiments were carried out independently at least three times. The results were expressed as means ± s.d.

Soft agar colony formation assay

1.2% Bacto-agar (BD Biosciences) was autoclaved and then warmed to 42°C. By mixing 1.2% agar with growth medium 1:1, 0.6% agar/medium was generated and then 1.5 ml of the mixture was quickly plated into 35mm dishes as base layer. Solidification was completed at room temperature for 45 min. Then $4.5x10^4$ cells were suspended in 3 ml growth medium supplemented with 3x serum and non-essential amino acids (NEAA, Thermo Fisher), then mixed with 1.5 ml 1.2% agar. The resulting mixture, 1 ml of cells/0.4% agar/medium (10,000 cells/ml) was quickly and gently added onto each plate for solidification. Formed colonies were examined using SZX-16 microscope and analyzed by CellSens Dimension software (Olympus, Shinjuku, Tokyo, Japan).

Transwell cell migration and invasion assays

Cells were starved in serum-free DMEM for 24h before the experiment. Then cells were harvested, washed and counted. Appropriate amounts of pre-warmed medium (no serum or 10% FBS) was added to the wells, then the inserts were carefully put into these wells using sterile forceps (for migration assays, we used Corning 8.0um PET track-etched membrane, 24 or 12 well format; for invasion assays, we used Corning Biocoat Matrigel Invasion Chamber, 24 well format). Then 1x10^5 cells (for 24 well plates) or 5x10^5 cells (for 12 well plates) in serum-free DMEM were added to the inserts. After 48h incubation, cells migrated through the membrane were stained with 0.5% crystal violet and cells not migrated through were removed using cotton swab. The stain was dissolved in 0.1M Sodium Citrate and the absorbance was read at 540nm on a plate reader.

Scratch wound healing assay

1x10^6 cells per well were placed in a 6-well plate. The "wound" was made by scratching the confluent monolayer across the well using a 200 µl pipette tip. At each time point, closure of the gap was recorded by taking pictures. Then the width of the gap was measured and normalized with 0 h.

Mammosphere formation assay

The mammosphere assay was developed as an approach to propagate mammary epithelial stem cells [47]. This assay was performed according to an online protocol (http://www.bio-protocol.org/e325). Briefly, tumorsphere medium was made by adding 20ng/ml epidermal growth factor (EGF), 10ng/ml basic fibroblast growth factor (bFGF), 5ug/ml Insulin and 0.4% Bovine Serum Albumin in DMEM/F12 (50/50) medium, and B27 supplement (Thermo Fisher) was freshly added to tumorsphere medium. Cells were collected, washed and counted followed by resuspending in tumorsphere medium with B27 supplement at a final concentration of 10,000 cells/ml. Then 2 ml cells were added to each well of an ultra-low attachment 6-well plate (Corning). After 7-day incubation, pictures of each well were taken and colonies were quantified using CellSens Dimension software. Secondary or tertiary mammospheres were generated by digesting primary mammospheres or secondary mammospheres and were seeded at the same density as primary mammospheres. All experiments were performed three times and bars represent the means of three independent experiments ± s.d.

Flow cytometry analysis

1x10^6 cells were collected and stained with antibodies or isotypes for 30 min on ice. Stained cells were washed with FACS buffer (PBS with 2% FBS) followed by fixing in 4% Paraformaldehyde (PFA) for 20 min. Fixed cells were then suspended in FACS buffer and analyzed on the LSR II XW4400 workstation (BD Biosciences).

Animal studies

4-5-week-old female BALB/c nu/nu athymic nude mice (Envigo, Madison, WI) were implanted with 3×10^6 MDA-MB-231 cells transfected with empty vector (n = 17) or LSD2 expression vector (n = 16) into the mammary fat pad. Tumor volumes were regularly assessed every two days by measuring 0.5 × length (mm) × width (mm) × width (mm). Mice were also weighed every two days. At the end of study, tumor or lung tissues of animals were collected and fixed with 4% paraformaldehyde. Tissues were processed into paraffin sections, and then subjected to hematoxylin-eosin (H&E) staining at the histological core facility at Magee Womens Research Institute.

Statistical analysis

Data were represented as the mean ±SD or ±SEM of three independent experiments. Two-tailed Student's t-test was used to determine the quantitative variables. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). P-values < 0.05 were considered statistically significant for all tests.

Abbreviations

KMT, histone lysine methyltransferase; KDM, histone lysine demethylases; LSD1/KDM1A, histone lysine-specific demethylase 1/1A; LSD2/KDM1B, histone lysine-specific demethylase 2/1B; TCGA, The Cancer Genome Atlas; AML, acute myeloid leukemia; DNMT, DNA methyltransferase; TNBC, triple-negative breast cancer; BCSC, breast cancer stem cell; ALDH, Aldehyde Dehydrogenase; MET, mesenchymal-epithelial transition; HDAC histone deacetylase; NuRD, nucleosome remodeling and histone deacetylase; PCNA, proliferating cell nuclear antigen; ESC, embryonic stem cell; CSC, cancer stem cell

Author contributions

LC, SNV and YH conceptualized and designed the experiments. YH, NED and OS provided funding support. LC, SNV, TPK, YQ and HW performed all experimental

procedures related to the study. NT and CC provided technical support. LC, SNV, KL and YH analyzed the data. LC and YH wrote the manuscript. SNV, SO and NED edited it. All authors contributed to the data analysis during discussions at joint meetings.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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